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NOCICEPTIVE NEURON-SPECIFIC CALCIUM CHANNEL ISOFORM AND USES THEREOF

Related Applications

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application serial number 60/443,474, filed January 29, 2003, the disclosure of which is incorporated by reference herein.

Government Support

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Field of the Invention

The invention pertains to N-type calcium channel subunit isoforms that are preferentially expressed in nociceptive neurons.

Background of the Invention

Nociceptive neurons in the dorsal root ganglia (DRG) respond to a wide variety of stimuli including heat, protons, and capsaicin (McCleskey and Gold, 1999). Nociceptive neurons have been identified based on a number of criteria including morphological, biophysical, and molecular properties. Capsaicin-responsiveness has been used as an important functional indicator of a sub-set of nociceptors that are also heat sensitive (Caterina and Julius, 2001; McCleskey and Gold, 1999).

Voltage gated calcium channels, also known as voltage dependent calcium channels, are multisubunit membrane spanning proteins which permit controlled calcium influx from an extracellular environment into the interior of a cell. Several types of voltage gated calcium channel have been described in different tissues, including N-type, P/Q-type, L-type and T-type channels. A voltage gated calcium channel permits entry into the cell of calcium upon depolarization of the membrane of the cell, which is a lessening of the difference in electrical potential between the outside and the inside of the cell.

Voltage-gated calcium (Ca) channels expressed in nociceptive neurons are present at presynaptic nerve terminals in the dorsal horn of the spinal cord where they regulate transmitter release. Consistent with this, the selective inhibitor of the N-type calcium channel, MVIIA which is also called SNX 111 or ziconotide, is a powerful analgesic (Bowersox et al., 1996; Brose et al., 1997; Chaplan et al., 1994; Cox, 2000). Further, mice lacking the N-type $Ca_V2.2$ subunit have higher pain thresholds compared to wild type (Hatakeyama et al., 2001; Kim et al., 2001; Saegusa et al., 2001; Saegusa et al., 2002). The N-type channel is also present at numerous other synapses and its importance in maintaining sympathetic tone is a major complication in analgesic therapy with N-type channel blockers (Ino et al., 2001; Miljanich and Ramachandran, 1995; Vanegas and Schaible, 2000). Consequently, there is considerable interest in establishing whether nociceptive neurons express a unique class of N-type channel. This possibility has gained momentum with growing appreciation that the $Ca_V2.2$ gene, which encodes the functional core of the N-type Ca channel, is subject to extensive alternative splicing that in some cases is tissue-specific (Lipscombe et al., 2002).

Previous studies have compared calcium currents in nociceptive and non-nociceptive neurons distinguished on the basis of various criteria (Blair and Bean, 2002; Cardenas et al., 1995; Petruska et al., 2000; Regan et al., 1991; Scroggs and Fox, 1992a). The most consistent difference reported is the presence of a larger low threshold T-type calcium current in nonnociceptive compared to nociceptive neurons. Recent studies suggest that the $Ca_V3.2$ T-type channel localizes to mechanoreceptors (Shin et al., 2003). Differences in high threshold, N-type currents of nociceptive compared to non-nociceptive neurons have not been reported.

Molecular analyses of RNA isolated from dorsal root ganglia have, nonetheless, provided evidence for the presence of multiple splice forms of the N-type $Ca_V2.2$ subunit that differ in their intracellular domains, and S3-S4 extracellular linkers (Lin et al., 1997; Lin et al., 1999; Lu and Dunlap, 1999; Pan and Lipscombe, 2000). None of the splice forms of $Ca_V2.2$ identified to date are exclusively expressed in dorsal root ganglia (Lipscombe et al., 2002).

Therefore, there is a need for the identification of splice isoforms of N-type calcium channel subunits that are preferentially or specifically expressed in nociceptive neurons. Identification of such differential expression of isoforms would permit the development of therapeutics which are specific for the distinct isoforms, thereby lessening side effects

resulting from the use of therapeutics which are effective for more than one calcium channel isoform.

Summary of the Invention

Based upon RT-PCR expression analysis, functional screening for capsaicin-responsiveness, and biophysical analysis at the single cell level, we present herein the identification of a novel splice isoform of the $Ca_V2.2\alpha$ subunit of N-type calcium channels, which is preferentially expressed in a sub-set of nociceptive neurons, and which contains exon 37a. The subunit is referred to herein as "$Ca_V2.2$e[37a]". The presence of exon 37a in $Ca_V2.2$ correlates with significantly larger N-type currents in neurons and non-neuronal expression systems.

We report the preferential expression of a splice variant of the $Ca_V2.2$ calcium channel in neurons sensitive to capsaicin. Given that distribution, it is clear that a screening assay for compounds that would preferentially block pain receptors can be established by comparing the compounds' activity on the $Ca_V2.2$ exon 37a variant and the $Ca_V2.2$ exon 37b variant, the latter being the control. Most ideally, the screening would be carried out in vitro on neurons genetically engineered to express either the 37a or 37b variant and acrivity of the compounds would be measured as a reduction in calcium current.

The invention provides isolated nucleic acid molecules that encode $Ca_V2.2$e[37a], fragments of those molecules, expression vectors containing the foregoing, and isolated host cells transfected with those molecules. The invention also provides isolated $Ca_V2.2$e[37a] polypeptides and fragments thereof, as well as inhibitors of the foregoing nucleic acids and polypeptides which modulate voltage-gated calcium influx. The foregoing can be used in the diagnosis or treatment of conditions characterized by increased or decreased N-type calcium channel activity correlated with the presence of the $Ca_V2.2$e[37a] subunit and can be used in methods to identify molecules that are therapeutically useful to modulate N-type calcium activity correlated with the presence of the $Ca_V2.2$e[37a] subunit, most particularly for treatment of pain (e.g., neuropathic pain), by modulating voltage regulated calcium influx.

The invention involves in one aspect recombinantly expressed $Ca_V2.2$ nucleic acids and polypeptides which include exon 37a or the amino acid sequence encoded thereby (CCRIHYKDMYSLLRCIAPPVGLGKNCPRRLAYK, SEQ ID NO:45). The first amino acid (Cys) in the amino acid sequence set forth as SEQ ID NO:45 is partly encoded by the end

of exon 36 and partly encoded by beginning of exon 37a, as the reading frame is not aligned to the exon-exon junctions. The amino acid sequence set forth as SEQ ID NO:45 is identical in human, mouse and rat. The nucleotide sequence that encodes SEQ ID NO:45 is identical in human and rat, but not in mouse (which therefore has a degenerate nucleotide sequence).

In one embodiment, the polypeptide comprises the amino acid sequence of SEQ ID NO:45, and preferably consists of the amino acid sequence of SEQ ID NO:45. In another embodiment the $Ca_V2.2$ polypeptide is a fragment or variant of the foregoing polypeptides, wherein the fragment or variant includes additions, deletions or substitutions of the amino acid sequence of SEQ ID NO:45 which confer the same function as SEQ ID NO:45. Preferred variants include those having additions, substitutions or deletions relative to the $Ca_V2.2e[37a]$ polypeptide sequence disclosed herein, particularly those variants which retain one or more of the activities of the $Ca_V2.2e[37a]$, including subunits with various other splice variations.

According to another aspect of the invention, isolated cells that recombinantly express an N-type calcium channel comprising a $Ca_V2.2$ subunit that comprises exon e37a ($Ca_V2.2e[37a]$) are provided. In some embodiments, the $Ca_V2.2e[37a]$ subunit has a human sequence, a mouse sequence or a rat sequence. In preferred embodiments, the cell is a neuron or an oocyte.

According to still another aspect of the invention, isolated neurons that express an N-type calcium channel comprising a $Ca_V2.2$ subunit that comprises exon e37a ($Ca_V2.2e[37a]$) are provided. In some embodiments, the neuron further expresses a marker of nociceptive neurons. Preferably the marker of nociceptive neurons is $Na_V1.8$ or vanilloid receptor VR1. In particularly preferred embodiments, the neuron expresses both $Na_V1.8$ and vanilloid receptor VR1.

In another aspect of the invention, methods are provided for identifying lead compounds for a pharmacological agent useful in the treatment of disease associated with increased or decreased voltage regulated calcium influx mediated by a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit. The methods include providing a cell or other membrane-encapsulated space comprising a $Ca_V2.2e[37a]$ polypeptide; contacting the cell or other membrane-encapsulated space with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of voltage regulated calcium influx into the cell or other membrane-encapsulated space; and determining

a test amount of voltage regulated calcium influx as a measure of the effect of the lead compounds for a pharmacological agent on the voltage regulated calcium influx mediated by a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit. A test amount of voltage regulated calcium influx which is less than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces voltage regulated calcium influx. A test amount of voltage regulated calcium influx which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases voltage regulated calcium influx. In some embodiments, the methods also include a step of loading the cell or other membrane-encapsulated space with a calcium-sensitive compound which is detectable in the presence of calcium, wherein the calcium-sensitive compound is detected as a measure of the voltage regulated calcium influx.

In a preferred embodiment, the pharmacological agent that specifically reduces voltage regulated calcium influx mediated by a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit is an agent that reduces N-type calcium channel current densities in nociceptive neurons. In another preferred embodiment, the pharmacological agent that specifically reduces voltage regulated calcium influx mediated by a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit is useful as an analgesic agent.

Methods for identifying compounds which selectively or preferentially bind a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit also are provided in another aspect of the invention. The methods include providing a first cell or membrane encapsulated space which expresses a N-type calcium channel that contains a $Ca_V2.2e[37a]$ subunit, providing a second cell or membrane encapsulated space which expresses a N-type calcium channel that does not contain a $Ca_V2.2e[37a]$ subunit, wherein the second cell or membrane encapsulated space is identical to the first cell except for the N-type calcium channel expressed, contacting the first cell or membrane encapsulated space and the second cell or membrane encapsulated space with a compound, and determining the binding of the compound to the first cell or membrane encapsulated space and the second cell or membrane encapsulated space. A compound which binds the first cell or membrane encapsulated space but does not bind the second cell or membrane encapsulated space is a compound which selectively binds the N-type calcium channel that contains a $Ca_V2.2e[37a]$ subunit. A compound which binds the first cell or membrane encapsulated space in an amount greater than the compound binds the second cell

or membrane encapsulated space is a compound which preferentially binds the N-type calcium channel that contains a $Ca_V2.2e[37a]$ subunit. In a preferred embodiment, the N-type calcium channel that does not contain a $Ca_V2.2e[37a]$ subunit is a N-type calcium channel that contains a $Ca_V2.2e[37b]$ subunit.

In a further aspect of the invention, methods for identifying compounds which selectively or preferentially bind to a $Ca_V2.2e[37a]$ isoform are provided. The methods include providing a $Ca_V2.2e[37a]$ isoform polypeptide or nucleic acid, providing a $Ca_V2.2e[37b]$ isoform polypeptide or nucleic acid, contacting the $Ca_V2.2e[37a]$ isoform polypeptide or nucleic acid and the $Ca_V2.2e[37b]$ subunit isoform polypeptide or nucleic acid with a compound, and determining the binding of the compound to the $Ca_V2.2e[37a]$ isoform polypeptide or nucleic acid and the $Ca_V2.2e[37b]$ isoform polypeptide or nucleic acid. A compound which binds the $Ca_V2.2e[37a]$ isoform polypeptide or nucleic acid but does not bind the human N-type calcium channel $Ca_V2.2e[37b]$ isoform polypeptide or nucleic acid is a compound which selectively binds the $Ca_V2.2e[37a]$ isoform, and wherein a compound which binds the $Ca_V2.2e[37a]$ isoform polypeptide or nucleic acid in an amount greater than the compound binds the $Ca_V2.2e[37b]$ isoform polypeptide or nucleic acid is a compound which preferentially binds the $Ca_V2.2e[37a]$ isoform.

In the foregoing methods for identifying compounds which selectively or preferentially bind to a $Ca_V2.2e[37a]$ isoform, the compound preferably is an antibody or a antigen-binding fragment thereof, a nucleic acid molecule or one or more compounds from a library of molecules. Preferably the library is a natural product library or a library generated by combinatorial chemistry.

According to yet another aspect of the invention, methods for preparing an analgesic agent are provided. The methods include identifying an agent that selectively or preferentially reduces calcium channel current densities in nociceptive neurons mediated by N-type calcium channels containing a $Ca_V2.2e[37a]$ subunit, and formulating the agent for administration to a subject in need of such treatment. The methods also can include identifying a compound according to the foregoing methods, and formulating the compound for administration to a subject in need of such treatment.

In another aspect, the invention provides double stranded RNA molecules specific for $Ca_V2.2e[37a]$ RNA. Preferably the double stranded RNA molecule is a siRNA molecule useful in RNA interference. Preferred embodiments include double stranded RNA molecules

that are 21-23 nucleotides in length and/or that have a 3' overhang. Preferably the 3' overhang is 2 nucleotides in length. In still other embodiments, the double stranded RNA molecule is a single molecule that comprises a hairpin structure.

In a further aspect of the invention, methods for inhibiting calcium influx in a neuronal cell mediated by a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit are provided. The methods include contacting the neuronal cell with an amount of a $Ca_V2.2e[37a]$ inhibitor effective to inhibit calcium influx in the mammalian cell. In some embodiments, the inhibitor is selected from the group consisting of an antibody which selectively binds the $Ca_V2.2e[37a]$ polypeptide, an antisense nucleic acid that reduces expression of a $Ca_V2.2e[37a]$ polypeptide, a siRNA that reduces expression of a $Ca_V2.2e[37a]$ polypeptide.

According to another aspect of the invention, methods for treating a subject afflicted by pain mediated by a N-type calcium channel containing a $Ca_V2.2e[37a]$ subunit are provided. The methods include administering to a subject in need of such treatment an inhibitor of the $Ca_V2.2e[37a]$ polypeptide in an amount effective to inhibit voltage regulated calcium influx and thereby to reduce the pain. In some embodiments, the inhibitor is selected from the group consisting of an antibody which selectively binds the $Ca_V2.2e[37a]$ polypeptide, an antisense nucleic acid that reduces expression of a $Ca_V2.2e[37a]$ polypeptide, a siRNA that reduces expression of a $Ca_V2.2e[37a]$ polypeptide.

In the foregoing methods, the inhibitor is administered prophylactically to a subject at risk of being afflicted with pain. Preferably the pain is neuropathic pain.

Use of the foregoing compositions in the preparation of a medicament, and particularly in the preparation of a medicament for the treatment of stroke, pain (e.g., neuropathic pain), traumatic brain injury, or a condition which results from excessive or insufficient voltage regulated calcium influx, is provided.

These and other aspects of the invention are described in greater detail below.

Brief Description of the Figures

Fig. 1 shows RT-PCR analysis of e37a and e37b splice variants.

Fig. 2 shows RT-PCR analysis of e37a and e37b in single DRG neurons.

Fig. 3 shows single cell RT-PCR (scRT-PCR) analysis of $Ca_V2.2$ e37a and e37b in capsaicin-responsive and capsaicin-non-responsive neurons.

Fig. 4 shows the experimental protocol used in various experiments.

Fig. 5 shows whole cell calcium currents in capsaicin-responsive and capsaicin-non-responsive neurons.

Fig. 6 shows that ω-Ctx GVIA-sensitive calcium currents in capsaicin-responsive and capsaicin-non-responsive neurons.

Fig. 7 shows that LVA currents rundown significantly over a 5 minute time period in capsaicin-non-responsive neurons.

Fig. 8 shows ω-Ctx GVIA-sensitive calcium currents in capsaicin-responsive neurons that contain and lack e37a.

Fig. 9 shows competitive RT-PCR analysis of e37a and e37b in whole tissue and single neurons.

Figure 10 shows that multiple splice forms of $Ca_V2.2$ are expressed in dorsal root ganglia. Fig. 10a, Putative membrane topology of the $Ca_V2.2$ subunit. The approximate location of constitutively expressed exons (horizontal black lines) and alternatively spliced exons, e18a, e24a, e31a and e37a/e37b (blue circles) are shown. Fig. 10b, RT-PCR analysis of e18a, e24a, and e31a in mRNA isolated from rat DRG. Primers flanked each splice site and generated the following products: 227 and 290 bp for Δe18a and +e18a; 114 and 126 bp for Δe24a and +e24a; and 169 and 175 bp for Δe31a and +e31a. PCR-derived cDNA products were separated on a 2 % agarose (e18a) or 4 % Metaphor agarose gel (e24a and e31a). Results are consistent with previous analyses of these sites of alternative splicing by RT-PCR and ribonuclease protection assays (Lin et al., 1997; Lin et al., 1999; Pan and Lipscombe, 2000).

Figure 11 shows that capsaicin-responsiveness in DRG neurons is correlated with the presence of VR1. DRG neurons were screened for capsaicin-responsiveness by whole cell recording (n = 269 cells). Whole cell currents recorded from Fig. 11a, a non-responsive neuron and Fig. 11b, a capsaicin-responsive neuron. The membrane potential was voltageclamped at -60 mV. The horizontal bar indicates the time and duration of capsaicin application (2 μM). No inward current was detected in 141 neurons. Inward currents were induced in 128 neurons during capsaicin challenge, with an average amplitude of 986 ± 118 pA. Fig. 11c, PCR-derived cDNA products amplified in two sets of reactions from 5 individual neurons (lanes 1-5) using VR1 and GAPDH -specific primers. The predicted size of PCR products was 125 bp and 274 bp, respectively. The capsaicin-responsiveness of each cell is indicated between gels (+ or -). Fig. 11d, Histogram showing the percentage of non-responsive cells (gray) and capsaicin-responsive cells (red) containing VR1. PCR products were amplified in 89 % of capsaicin-responsive cells (25 of 28) with VR1 primers compared to 13 % of non-responsive cells (2 of 15).

Figure 12 shows that expression patterns of exons, e18a, e24a, and e31a, do not correlate with capsaicin-responsiveness. Representative gels showing single cell RT-PCRderived cDNA products amplified using $Ca_V2.2$-specific primers flanking exons Fig. 12a, e18a; Fig. 3b, e24a; and Fig. 12c, e31a, together with histograms summarizing the distribution of exons based on capsaicin-responsiveness. Control GAPDH-specific primers are used in each single cell reaction. Products amplified from four cells are shown for each primer pair (lanes 1-4). In Fig. 12c, the first two lanes show products amplified from $Ca_V2.2e[\Delta e31a]$ and $Ca_V2.2e[+e31a]$ clones to establish that a 6 bp difference is resolvable in a 4 % Metaphor gel. Sizes of cDNA products were respectively, 227 bp and 290 bp for Δe18 and +e18a; 114 bp and 126 for Δe24a and +e24a; and 169 bp and 175 bp for .e31a and +e31a. Histograms show percent cells that lack the specified exon (Δ) and that express both splice isoforms lacking and containing the exon (both). Histograms separate cells based on capsaicin-non-responsiveness (gray) and capsaicin-responsiveness (red). The total number of cells analyzed is shown below each histogram. Capsaicin responsiveness of each cell is indicated between gels (+ or -).

Figure 13 shows that exon 37a is expressed exclusively in dorsal root ganglia. Fig. 13a, Splicing pattern of mutually exclusive exons e37b and e37a of $Ca_V2.2e$[37a] based on analysis of the public rat genomic sequence (accession number NW_043710) and our sequencing (accession number AY211499). Exons are denoted with solid bars and introns with horizontal lines. Exon lengths are 128, 97, 97, and 109 bps for e36, e37a, e37b, and e38 respectively (accession numbers AY211499 and AY211500). 37a amino acid sequence is CCRIHYKDMYSLLRCIAPPVGLGKNCPRRLAY (SEQ ID NO:46); 37b amino acid is sequence CGRISYNDMFEMLKHMSPPLGLGKKCPARVAY (SEQ ID NO:47) Fig. 13b, Expression pattern of e37b and e37a in RNA isolated from various regions of the adult rat nervous system. SCG, superior cervical ganglia; DRG, dorsal root ganglia; SC, spinal cord; MD, medulla; MB, midbrain; CM, cerebellum; TH, thalamus; HC, hippocampus; CX, cortex. Primers were exon-specific for e37a and e37b. PCR-derived products were separated on a 3 % agarose gel. Each lane contains equal amounts of PCR reaction. Figs. 13c and Fig. 13d, Levels of $Ca_V2.2$ mRNA containing e37a and e37b were estimated in P5 (Fig. 13c), and adult (Fig. 13d) DRG tissue by competitive RT-PCR. Each primer pair generated two PCR products, 108 bp from $Ca_V2.2$ cDNA and 135 bp from competitive template. Gel shows products amplified by RT-PCR of RNA isolated from whole DRG (500 pg per reaction = ~5 single cells) for e37a and e37b in the presence of serial dilutions of competitive template (10^{-18} to 10^{-22} M). In P5 tissue, Fig. 13c, the e37b competitive template product was completely depleted at $5x10^{-21}$ M by the tissue-derived e37b template. The two were approximately equal in intensity at $5x10^{-20}$ M. The e37a competitive template product was completely depleted at $5x10^{-22}$ M by the tissue-derived e37a template. The two were approximately equal in intensity at $5x10^{-21}$ M. In adult tissue, Fig. 13d, the e37b competitive template product was completely depleted at $1x10^{-21}$ M by the tissue-derived e37b template. The two were approximately equal in intensity at $5x10^{-20}$ M. The e37a competitive template product was completely depleted at $1x10^{-22}$ M by the tissue-derived e37a template. The two were approximately equal in intensity at $5x10^{-21}$ M. These gels are representative of three experiments that gave similar results.

Figure 14 shows that exon 37a is preferentially expressed in nociceptive neurons. Single neurons were analyzed by RT-PCR and the expression pattern of e37a correlated with capsaicin-responsiveness. Fig. 14a and Fig. 14b, Histogram summary showing the number of cells expressing e37b and e37a in capsaicin-non-responsive neurons (gray) and responsive

neurons (red). e37a-specific primers amplified products in 32 of 58 capsaicin-responsive and 5 of 27 non-responsive neurons. Fig. 14c, Histogram summary of the number of cells expressing e37a, $Na_V1.8$, and both e37a and $Na_V1.8$, in 24 capsaicin-responsive cells. Fig. 14d, Representative gels showing RT-PCR products amplified with e37a, e37b and GAPDH - specific primers from four single cells (lanes 1-4). The capsaicin-responsiveness of each cell is indicated between gels (+ or -). Fig. 14e, Gels showing RT-PCR products amplified with $Na_V1.8$, e37a, and GAPDH -specific primers from four neurons (lanes 1-4). The capsaicin-responsiveness of each neuron is indicated between gels (+ or -).

Figure 15 depicts a comparison of calcium channel currents in capsaicin-non-responsive and responsive neurons. Fig. 15a, Average, peak current-voltage relationships for whole cell calcium currents measured in capsaicin-responsive (●) and non-responsive (○) neurons of dorsal root ganglia. Average, peak current density and capacitance were, for capsaicin-responsive neurons: 135 ± 19 pA/pF and 18 ± 2 pF, n = 20; for capsaicin-non-responsive neurons: 123 ± 17 pA/pF and 27 ± 3 pF, n = 9. Curves are fit with the sum of two Boltzmann-GHK functions. Estimated $V_{½}$ values were ~-45 mV and ~-15 mV for low and high voltage-activated currents, respectively. *Upper inset*: Representative, low voltage-activated and high voltage-activated whole cell calcium currents activated by voltage steps to -40 mV and -5 mV, respectively, from a holding potential of -80 mV from a capsaicin-non-responsive neuron. *Lower inset*: Same as upper inset from a capsaicin-responsive neuron. Scale bars: 1 nA, 10 ms. Fig. 15b, Average, peak current voltage relationships for ω-Ctx GVIA-subtracted calcium current in capsaicin-responsive (●) and non-responsive (○) neurons. Average, peak current densities were 111 ± 12 pA/pF (n = 20) for capsaicin-responsive compared to 72 ± 8 pA/pF (n = 9) for non-responsive neurons. These values are significantly different ($p < 0.05$). The ω-Ctx GVIA-sensitive current was 71 ± 2% of the total whole cell calcium current in capsaicin-responsive neurons and 68 ± 2% of whole cell current in non-responsive neurons. Curves are fit with the sum of two Boltzmann-GHK functions. Average $V_{½}$ and k values were calculated from fits of individual N-type current-voltage relationships. In capsaicin-non-responsive cells, for the low voltage-activated component, $V_{½}$ and k values were -25 ± 4 mV and 4.8 ± 0.5 compared to -21 ± 2 mV and 6 ± 0.6 for capsaicin-responsive cells. In capsaicin non- responsive neurons average $V_{½}$ and k values were, for the high voltage-activated component: -16 ± 2 mV and 5.4 ± 0.6 compared to -15 ±

1 mV and 5.2 ± 0.3 for capsaicin-responsive cells. Values of $V_{½}$ and k were not significantly different between capsaicin-responsive and capsaicin-non-responsive neurons ($p > 0.05$). *Inset,* Representative ω-Ctx GVIA-sensitive current recorded at -5 mV from a capsaicin-responsive neuron (lower trace) and non-responsive neuron (upper trace). Scale bar: 25 pA/pF, 10 ms. Data are mean ± se.

Figure 16 show that exon 37a expression is associated with larger N-type currents in capsaicin-responsive neurons. Fig. 16a, Average, peak current-voltage relationships of ω-Ctx GVIA-sensitive calcium current in capsaicin-responsive neurons that contain (•) and lack (○) e37a. Average peak current density at 0 mV and capacitance of responsive neurons that contain e37a were 122 ± 11 pA/pF and 20 ± 3 pF (n = 8) compared to 76 ± 3 pA/pF and 18 ± 1 pF for neurons that lack e37a (n = 8). Peak current densities are significantly greater in neurons containing e37a ($p < 0.05$). Current densities were significantly different between splice isoforms when compared at -10 mV, –5 mV, 0 mV, + 5 mV, and +10 mV ($p < 0.05$). Curves are Boltzmann-linear IV fits. Average $V_{½}$ and k values are –12.7 ± 1.8 mV and 4.6 ± 0.4, n = 8, for neurons containing e37a compared to –13.6 ± 1.7 mV and 5.4 ± 0.3, n = 8, for neurons lacking e37a. $V_{½}$ and k values are not significantly different between the two groups ($p > 0.05$). Inset shows examples of toxin-subtracted currents from neurons containing (•) and lacking (○) exon 37a. Scale bars are 10 ms and 20 pA/pF. Fig. 16b, Averages of time constants estimated from fits of the activation phase of toxin-subtracted N-type currents induced by step depolarizations to indicated test potentials, from capsaicin-responsive neurons containing (•) and lacking (○) exon 37a. Fig. 16c, Average time constants estimated from fits of the inactivation kinetics of toxin-subtracted N-type currents induced by step depolarizations to indicated test potentials, from capsaicin-responsive neurons containing (•) and lacking (○) exon 37a. Fig. 16d, Representative gels showing RT-PCR products amplified from four single cells (lanes 1-4) with primers specific for e37a, e37b, and GAPDH. Cells were used in the analysis shown in Fig. 16a. Data are mean ± se.

Figure 17 shows that $Ca_V2.2e[37a]$ clones induce N-type currents in Xenopus oocytes that are significantly larger compared to $Ca_V2.2e[37b]$. Fig. 17a, Average peak current-voltage relationships in oocytes expressing $Ca_V2.2e[37a]$ ( ) and $Ca_V2.2e[37b]$ (○). After 5 days post injection, average $Ca_V2.2e[37a]$ peak currents were 211 ± 2 nA (n = 8) compared to

134 ± 4 nA for $Ca_V2.2e[37b]$ (n = 8). Peak $Ca_V2.2e[37a]$ currents were significantly greater than $Ca_V2.2e[37b]$ at day 4, 5 and 6 after injection ($p < 0.05$). The dotted line shows the predicted current voltage-relationship of $Ca_V2.2e[37b]$ calculated using the Boltzmann activation curve of $Ca_V2.2e[37a]$ shown in Fig. 17b. This predicted curve demonstrates that an 8 mV left shift in voltage-dependence of channel activation (see Fig. 17b) is insufficient to account for the significantly larger currents of $Ca_V2.2e[37a]$ compared to $Ca_V2.2e[37b]$. *Inset*: Representative $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$ currents induced by step depolarizations to peak current (-5 mV for $Ca_V2.2e[37a]$ and 0 mV for $Ca_V2.2e[37b]$) from a holding potential of -80 mV. Scale bar: 50 nA, 20 ms. $V_{½}$ and k values were estimated from Boltzmann-GHK fits to individual data sets. Average $V_{½}$ values are -17.9 ± 0.6 mV, n = 8, for $Ca_V2.2e[37a]$ and -9.7 ± 0.4 mV, n = 8, for $Ca_V2.2e[37b]$. k values are 5.3 ± 0.1 for $Ca_V2.2e[37a]$ and 5.1 ± 0.1 for $Ca_V2.2e[37b]$. Average, macroscopic activation time constants τ_{act} are 7.2 ± 0.5 ms for $Ca_V2.2e[37a]$, n = 8, and 10.6 ± 0.5 ms for $Ca_V2.2$ e[37b], n = 9. These values are significantly different ($p < 0.05$). Peak currents in oocytes expressing $Ca_V2.2e[37a]$ were 186 ± 2 nA (n = 4), 211 ± 2 nA (n = 8), and 387 ± 20 nA (n = 8) at days 4, 5 and 6 days post injection, respectively. Compared to 68 ± 2 nA (n = 3), 134 ± 2 nA (n = 8), and 204 ± 10 nA (n = 8) at 4, 5 and 6 days post injection, respectively, in oocytes expressing $Ca_V2.2e[37b]$. In all cases values between splice isoforms were significantly different on a given day ($p < 0.05$). Fig. 17b, Normalized, averaged activation curves for N-type currents in oocytes expressing $Ca_V2.2e[37a]$ (●) and $Ca_V2.2e[37b]$ (○). Curves were generated from slope conductances calculated from peak current-voltage relationships shown in Fig. 8a, and assuming a reversal potential of + 40 mV. Boltzmann functions were fit to individual curves and used to calculate average values for $V_{½}$ and k. These were for $Ca_V2.2e[37a]$: -19.7 ± 0.6 mV and 4.4 ± 0.2; and for $Ca_V2.2e[37b]$: -11.7 ± 0.5 mV and 4.7 ± 0.1. $V_{½}$ values are significantly different ($p < 0.05$); k values are not significantly different. Fig. 17c, Normalized, averaged steady-state inactivation curves for N-type currents in oocytes expressing $Ca_V2.2e[37a]$ (●) and $Ca_V2.2e[37b]$ (○). Curves were generated from peak currents elicited by 300 ms test pulses to -5 mV ($Ca_V2.2e[37a]$, n = 12) or 0 mV ($Ca_V2.2e[37b]$, n = 11) after 20 second conditioning prepulses to voltages ranging from -100 mV to +20 mV. Barium (5 mM) was the charged carrier. Peak currents are plotted as a fraction of the maximum current at the indicated holding potentials. $V_{½}$ and k values were estimated from Boltzmann fits to data from individual cells. Average $V_{½}$ and k values were

for $Ca_V2.2e[37a]$: -72.7 ± 0.8 mV and 8.1 ± 0.4; and for $Ca_V2.2e[37b]$: -72.0 ± 0.4 mV 8.1 ± 0.6. Values are not significantly different. Inactivation kinetics were also measured, $Ca_V2.2e[37a]$: $\tau_{inact-1}$ = 393 ± 17 ms and $\tau_{inact-2}$ = 89 ± 5 ms compared to 384 ± 8 ms and 82 ± 2 ms for $Ca_V2.2e[37b]$. Values are not significantly different between splice isoforms. These data are representative of four separate injections. Data are mean ± se.

Detailed Description of the Invention

The present invention in one aspect involves the discovery that a particular splice isoform of the N-type calcium channel $Ca_V2.2\alpha$ (gene CACNA1B) containing the amino acid sequence encoded by exon 37a, referred to herein as the $Ca_V2.2e[37a]$ isoform, is preferentially and specifically expressed in nociceptive neurons. The invention also pertains to the use of these isoforms in drug discovery to identify agents that selectively inhibit the function of N-type calcium channels containing a $Ca_V2.2e[37a]$ subunit, particularly in cell-based assays, and agents identified by such methods. The invention also includes agents that modulate expression of the $Ca_V2.2e[37a]$ gene transcript (transcription or translation), such as siRNA that selectively reduces expression of $Ca_V2.2e[37a]$, and it use in treating conditions that involve this subunit, particularly pain.

As used herein, $Ca_V2.2e[37a]$ refers to any N-type calcium channel $Ca_V2.2$ subunit that contains an amino acid sequence encoded by exon 37a, or nucleic acid molecules encoding such subunits. The human, mouse and rat $Ca_V2.2e$ exon 37a-encoded amino acid sequence is presented as SEQ ID NO:45; the equivalent exon in other species can be identified by routine experimental procedures including homology analysis of a species' genomic sequence, transcript sequences (e.g., mRNA), etc. as will be understood by those of skill in the art. N-type calcium channel $Ca_V2.2$ subunits that do not contain exon 37a have been deposited in GenBank under the following accession numbers.

Table 1: Species Homologs

Species	Accession number	SEQ ID NO
Rattus norvegicus	AF055477	27, 28
	M92905	29, 30
Homo sapiens	M94172	31, 32
	M94173	33, 34
Mus musculus	AF042317	37, 38
	U04999	39, 40
Oryctolagus cuniculus (rabbit)	D14157	41, 42
Bos taurus	AF173882	43, 44

The human nucleotide sequence of exon 37a (then called exon 42) was identified and deposited under accession number AF238295; it is provided here as SEQ ID NO:35. The amino acid sequence encoded by this sequence is provided here as SEQ ID NO:36. The amino acid sequence (YDPAACCRIHYKDMYSLLRCIAPPVGLGKNCPRRLAYK; SEQ ID NO:36) includes amino acids (double underline) encoded by the 3' end of exon 41 (nucleotides 1-17) and amino acids (underlined) encoded by exon 42 (nucleotides 4510-4606). Note that amino acid 6 (Cys) is partly encoded by both exon nucleotide sequences.

Because the present $Ca_V2.2e[37a]$ isoform is a splice variant of the N-type calcium channel $Ca_V2.2$ subunit, it is apparent that the invention is meant to embrace N-type calcium channel $Ca_V2.2$ subunit variants which vary by alternative splicing of sequences other than the exon 37a insert (SEQ ID NO:45), and nucleotide sequence encoding such polypeptides. For example, the invention embraces the splice variant polypeptides, and nucleic acid molecules encoding such splice variant polypeptides, as described in US Patent 6,353,091. As shown in the Examples, the $Ca_V2.2e[37a]$ subunit is significantly and differentially expressed in certain subsets of neurons that exert different function in different parts of the brain. This opens the possibility for the selective treatment of disorders which involve those parts of the brain.

The invention involves in one aspect $Ca_V2.2e[37a]$ nucleic acids and polypeptides, as well as therapeutics relating thereto. The invention also embraces isolated functionally equivalent variants, useful analogs and fragments of the foregoing nucleic acids and

polypeptides; complements of the foregoing nucleic acids; and molecules which selectively bind the foregoing nucleic acids and polypeptides.

The $Ca_V2.2e[37a]$ nucleic acids and polypeptides of the invention are isolated. The term "isolated", as used herein in reference to a nucleic acid molecule, means a nucleic acid sequence: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and electrophoretic or chromatographic separation. The term "isolated", as used herein in reference to a polypeptide, means a polypeptide encoded by an isolated nucleic acid sequence, as well as polypeptides synthesized by, for example, chemical synthetic methods, and polypeptides separated from biological materials, and then purified, using conventional protein analytical or preparatory procedures, to an extent that permits them to be used according to the methods described herein.

As used herein a $Ca_V2.2e[37a]$ nucleic acid refers to an isolated nucleic acid molecule which codes for a $Ca_V2.2$ subunit protein that includes exon 37a. $Ca_V2.2e[37a]$ nucleic acids are those nucleic acid molecules which code for $Ca_V2.2e[37a]$ polypeptides which include the sequence of SEQ ID NO: 45. The $Ca_V2.2e[37a]$ nucleic acids of the invention also include alleles and species homologs of the foregoing nucleic acids, as well as fragments of the foregoing nucleic acids, provided that the allele, species isoform or fragment contains exon 37a (i.e., the pertinent exon of the particular species that is homologous to those described herein). Such fragments can be used, for example, as probes in hybridization assays, as primers in a polymerase chain reaction (PCR), and for reconbination with exon 37b containing nucleic acids. In a preferred embodiment, exon 37a-specific fragments and their complements can be used in the preparation of double stranded RNA molecules (such as siRNA molecules and hairpins) useful for inhibition of expression by RNA interference (RNAi). Preferred $Ca_V2.2e[37a]$ nucleic acids include nucleotides 4510-4606 of SEQ ID NO:35 (Accession number AF238295). Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein "$Ca_V2.2e[37a]$ activity" refers to an ability of a molecule to modulate voltage regulated calcium influx. As shown in the Examples, neurons and other cells that express N-type calcium channels containing $Ca_V2.2e[37a]$ have significantly larger N-type currents. Thus, a molecule which inhibits $Ca_V2.2e[37a]$ activity (an antagonist) is one which inhibits voltage regulated calcium influx via this calcium channel and a molecule which

increases $Ca_V2.2e[37a]$ activity (an agonist) is one which increases voltage regulated calcium influx via this calcium channel. Changes in $Ca_V2.2e[37a]$ activity can be measured by changes in voltage regulated calcium influx by *in vitro* assays such as those disclosed herein, including patch-clamp assays, and other assays known to those of skill in the art, such as assays employing calcium sensitive fluorescent compounds such as fura-2.

Alleles of the $Ca_V2.2e[37a]$ nucleic acids of the invention can be identified by conventional techniques. For example, alleles of $Ca_V2.2e[37a]$ can be isolated by hybridizing a probe which includes nucleotides 4510-4606 of SEQ ID NO:35, or another nucleotide sequence that encodes SEQ ID NO:45 (except the first amino acid, which as noted herein is encoded partly by exon 36 and partly by exon 37a), under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for $Ca_V2.2e[37a]$ polypeptides and which hybridize to a nucleic acid molecule consisting of $Ca_V2.2$ exon 37a under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH_2PO_4(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.015M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 x SSC/0.1 x SDS at temperatures up to 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of alleles of $Ca_V2.2e[37a]$ nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and

sequencing.

In screening for $Ca_V2.2e$[37a] nucleic acids, nucleic acid amplification techniques such as PCR can be performed as known to one of ordinary skill in the art. In addition, hybridization techniques such as Southern and Northern blots may be performed using the foregoing stringent conditions, together with a labeled (e.g., fluorescently, radioactively) probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against various photon capture devices (e.g., phosphorimager) or X-ray film to detect the radioactive signal.

Alleles of $Ca_V2.2e$[37a] nucleic acids also can be identified using *in silico* techniques of database mining, sequence comparison, etc. as will be known to one of ordinary skill in the art.

The $Ca_V2.2e$[37a] nucleic acids of the invention also include degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating $Ca_V2.2e$[37a] polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code.

The invention also provides isolated fragments of $Ca_V2.2e$[37a] nucleic acids, which include at least part of the nucleotide sequence that encodes SEQ ID NO:45. The fragments can be used as probes in Southern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and every integer therebetween and are useful e.g. as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 100, 200, 250, 300, 400 or more

nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. Likewise, fragments can be employed to produce non-fused fragments of the $Ca_V2.2e[37a]$ polypeptides, useful, for example, in the preparation of antibodies, in immunoassays, and the like. The foregoing nucleic acid fragments further can be used as antisense molecules or siRNA molecules to inhibit the expression of $Ca_V2.2e[37a]$ nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

The invention also includes functionally equivalent variants of the $Ca_V2.2e[37a]$, which include variant nucleic acids and polypeptides that retain one or more of the functional properties of the $Ca_V2.2e[37a]$, such as the different current densities as described in greater detail below. For example, functionally equivalent variants include a $Ca_V2.2e[37a]$ molecule which has had a portion of the extracellular domain removed or replaced by a similar domain from another calcium channel α_1 subunit (e.g. a "domain-swapping" variant). Other functionally equivalent variants will be known to one of ordinary skill in the art, as will methods for preparing such variants. The activity of a functionally equivalent variant can be determined using the methods provided, e.g., herein, in Lin et al., *Neuron* 18:153-166, 1997, and in US patent 5,429,921. Such variants are useful, *inter alia*, in assays for identification of compounds which bind and/or regulate the calcium influx function of the $Ca_V2.2e[37a]$, and for determining the portions of the $Ca_V2.2e[37a]$ which are required for calcium influx activity.

Variants that are non-functional also can be prepared as described above. Such variants are useful, for example, as negative controls in experiments testing subunit activity, and as inhibition of nociceptive-specific N-type calcium channel activity.

A $Ca_V2.2e[37a]$ nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the $Ca_V2.2e[37a]$ nucleic acid within a eukaryotic or prokaryotic cell. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the $Ca_V2.2e[37a]$ nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine

phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, β-actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney murine leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined $Ca_V2.2e[37a]$ nucleic acid. The gene expression sequences optionally includes enhancer sequences or upstream activator sequences as desired.

The $Ca_V2.2e[37a]$ nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the $Ca_V2.2e[37a]$ coding sequence under the influence or control of the gene expression sequence. If it is desired that the $Ca_V2.2e[37a]$ sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the $Ca_V2.2e[37a]$ sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the $Ca_V2.2e[37a]$ sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a $Ca_V2.2e[37a]$ nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that $Ca_V2.2e[37a]$ nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The $Ca_V2.2e[37a]$ nucleic acid and the $Ca_V2.2e[37a]$ polypeptide (including the $Ca_V2.2e[37a]$ inhibitors described below) of the invention can be delivered to the eukaryotic or prokaryotic cell alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating: (1) delivery of a $Ca_V2.2e[37a]$ nucleic acid or polypeptide to a target cell or (2) uptake of a $Ca_V2.2e[37a]$ nucleic acid or polypeptide by a target cell. Preferably, the vectors transport the $Ca_V2.2e[37a]$ nucleic acid or polypeptide into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor (e.g. a receptor, an antigen recognized by an antibody) for the targeting ligand. In this manner, the vector (containing a $Ca_V2.2e[37a]$ nucleic acid or a $Ca_V2.2e[37a]$ polypeptide) can be selectively delivered to a specific cell. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are more useful for delivery/uptake of $Ca_V2.2e[37a]$ nucleic acids to/by a target cell. Chemical/physical vectors are more useful for delivery/uptake of $Ca_V2.2e[37a]$ nucleic acids or $Ca_V2.2e[37a]$ proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be attached to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; and polio virus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered

retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., *"Gene Transfer and Expression, A Laboratory Manual,"* W.H. Freeman C.O., New York (1990) and Murry, E.J. Ed. *"Methods in Molecular Biology,"* vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a $Ca_V2.2e[37a]$ polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as the pcDNA series of vectors (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector

(Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1α, which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992).

In addition to the biological vectors, chemical/physical vectors may be used to deliver a $Ca_V2.2e[37a]$ nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated $Ca_V2.2e[37a]$ nucleic acid or polypeptide to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vesicles which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2 - 4.0 μ can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, v. 6, p. 77 (1981)). In order for a liposome to be an efficient nucleic acid transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the nucleic acid of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to a particular cell will depend on the particular cell or tissue type. Additionally when the vector encapsulates a nucleic acid, the vector may be coupled to a nuclear targeting peptide, which will direct the $Ca_V2.2e[37a]$ nucleic acid to the

nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, V. 3, p. 235-241 (1985).

Other exemplary compositions that can be used to facilitate uptake by a target cell of the $Ca_V2.2e[37a]$ nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a $Ca_V2.2e[37a]$ nucleic acid into a preselected location within a target cell chromosome).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the $Ca_V2.2e[37a]$ cDNA sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., neurons, oocytes, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as human, pig, goat, primate, mouse, rat, etc., which can be used for the identification of molecules that regulate the function of $Ca_V2.2e[37a]$ selectively (e.g., by screening chemical compounds libraries) They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include neuronal cells including PC12 cells, *Xenopus* oocytes, bone marrow stem cells and embryonic stem cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

The invention also provides isolated $Ca_V2.2e[37a]$ polypeptides which include the amino acid sequence of SEQ ID NO:45, encoded by the $Ca_V2.2e[37a]$ nucleic acids described above. The preferred $Ca_V2.2e[37a]$ polypeptide has the amino acid sequence of any of the N-type calcium channel subunit proteins disclosed herein (see e.g., Table 1), with the amino acid sequence of exon 37a (SEQ ID NO:45) substituted for that of exon 37b. $Ca_V2.2e[37a]$

polypeptides also embrace alleles, isoforms (such as splice variants), functionally equivalent variants and analogs (those non-allelic polypeptides which vary in amino acid sequence from SEQ ID NO:45 by 1, 2, 3, 4, or 5 amino acids) provided that such polypeptides retain $Ca_V2.2e[37a]$ activity. Non-functional variants also are embraced by the invention; these are useful as antagonists of calcium channel function, as negative controls in assays, and the like. Such alleles, isoforms, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes such as to generate antibodies, or as a component of an immunoassay.

Fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the $Ca_V2.2e[37a]$ polypeptide, in particular voltage regulated calcium influx. Other functional capabilities which can be retained in an allele, isoform, variant, analog or fragment (generically called "variant" herein) of a $Ca_V2.2e[37a]$ polypeptide include interaction with antibodies and interaction with other polypeptides (such as other subunits of the human N-type calcium channel). Those skilled in the art are well versed in methods for selecting alleles, isoforms, variants, analogs or fragments which retain a functional capability of the $Ca_V2.2e[37a]$. Confirmation of the functional capability of the fragment can be carried out by synthesis of the fragment and testing of the capability according to standard methods. For example, to test the voltage regulated calcium influx of a $Ca_V2.2e[37a]$ fragment, one inserts or expresses the fragment in a cell in which calcium influx can be measured. Such methods, which are standard in the art, are described further in the examples.

The invention embraces variants of the $Ca_V2.2e[37a]$ polypeptides described above. As used herein, a "variant" of a $Ca_V2.2e[37a]$ polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a $Ca_V2.2e[37a]$ polypeptide. Modifications which create a $Ca_V2.2e[37a]$ variant can be made to a $Ca_V2.2e[37a]$ polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a $Ca_V2.2e[37a]$ polypeptide, such as voltage gated calcium influx; 2) to enhance a property of a $Ca_V2.2e[37a]$ polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; 3) to provide a novel activity or property to a $Ca_V2.2e[37a]$ polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 4) to establish that an amino acid substitution, such as a different sequence encoded by an alternatively spliced exon, does or does not affect voltage gated calcium influx..

Modifications to a $Ca_V2.2e[37a]$ polypeptide are typically made to the nucleic acid which encodes the $Ca_V2.2e[37a]$ polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the $Ca_V2.2e[37a]$ amino acid sequence, but always including the amino acid sequence encoded by exon 37a.

One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a variant $Ca_V2.2e[37a]$ according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87, 1997, whereby proteins can be designed *de novo*. Additional computer-based modeling methods also are available. The method can be applied to a known protein to vary a only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of a $Ca_V2.2e[37a]$ polypeptide can be proposed and tested to determine whether the variant retains a desired conformation.

Variants include $Ca_V2.2e[37a]$ polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a $Ca_V2.2e[37a]$ polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a $Ca_V2.2e[37a]$ polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis (e.g., directed molecular evolution, optionally of a selected site) in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant $Ca_V2.2e[37a]$ polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which

provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a $Ca_V2.2e[37a]$ gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of $Ca_V2.2e[37a]$ polypeptides can be tested by cloning the gene encoding the variant $Ca_V2.2e[37a]$ polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the variant $Ca_V2.2e[37a]$ polypeptide, and testing for a functional capability of the $Ca_V2.2e[37a]$ polypeptides as disclosed herein. For example, the variant $Ca_V2.2e[37a]$ polypeptide can be tested for ability to provide voltage regulated calcium influx, as set forth below in the examples. Preparation of other variant polypeptides may favor testing of other activities, as will be known to one of ordinary skill in the art.

The skilled artisan will also realize that conservative amino acid substitutions may be made in $Ca_V2.2e[37a]$ polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the $Ca_V2.2e[37a]$ polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the $Ca_V2.2e[37a]$ polypeptides include conservative amino acid substitutions of the $Ca_V2.2$ sequences described herein, but excluding the portion of the polypeptide consisting of the amino acid sequence encoded by exon 37a. Other variants can include amino acid substitutions of the exon 37a-encoded sequence, which may be useful for determining which amino acid residues are of importance in the functional activity of $Ca_V2.2e[37a]$ protein; such variants can be used in assays to screen molecules for $Ca_V2.2e[37a]$ antagonist or agonist activity as well. Exemplary conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of $Ca_V2.2e[37a]$ polypeptide to produce functionally equivalent variants of $Ca_V2.2e[37a]$ polypeptides typically are made by alteration of a nucleic acid sequence encoding $Ca_V2.2e[37a]$ polypeptides (e.g., nucleotides 5212-5308 of SEQ ID NO:23). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (*Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a $Ca_V2.2e[37a]$ polypeptide. Where amino acid substitutions are made to a small unique fragment of a $Ca_V2.2e[37a]$ polypeptide, such as a specific domain, the substitutions can be made by directly synthesizing the peptide. The activity of functionally equivalent variants of $Ca_V2.2e[37a]$ polypeptides can be tested by cloning the gene encoding the altered $Ca_V2.2e[37a]$ polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered $Ca_V2.2e[37a]$ polypeptide, and testing for the ability of the $Ca_V2.2e[37a]$ polypeptide to transduce voltage regulated calcium influx. Peptides which are chemically synthesized can be tested directly for function.

A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated $Ca_V2.2e[37a]$ molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce polypeptide. Those skilled in the art also can readily follow known methods for isolating $Ca_V2.2e[37a]$ polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

The invention also embraces agents which bind selectively to the $Ca_V2.2e[37a]$ molecules (having or encoding SEQ ID NO:45) and agents which bind preferentially to the $Ca_V2.2e[37a]$ (having or encoding SEQ ID NO:45) as well as agents which bind to variants and fragments of the polypeptides and nucleic acids as described herein. Selective binding means that the agent binds to the $Ca_V2.2e[37a]$ but not to N-type calcium channel non-

$Ca_V2.2e[37a]$ subunits (i.e., those subunits which do not have or encode SEQ ID NO:45). Preferential binding means that the agent binds more to the $Ca_V2.2e[37a]$ than to N-type calcium channel non-$Ca_V2.2e[37a]$ subunits, e.g., the agent binds with greater affinity or avidity to the $Ca_V2.2e[37a]$ having or encoding SEQ ID NO:45. The agents include polypeptides which bind to $Ca_V2.2e[37a]$, antisense nucleic acids, and siRNA molecules. The agents can inhibit or increase $Ca_V2.2e[37a]$ activity (antagonists and agonists, respectively).

Some of the agents are inhibitors. A $Ca_V2.2e[37a]$ inhibitor is an agent that inhibits $Ca_V2.2e[37a]$ mediated voltage gated calcium influx. $Ca_V2.2e[37a]$ inhibitors include dominant negative peptides and modified known N-type calcium channel inhibitors including modified ω-conotoxin peptides, such as analogs of ziconotide (SNX-111). Small organic molecule calcium channel inhibitors, such as fluspirilene, NNC 09-0026(-)-trans-1-butyl-4-(4-dimethylaminophenyl)-3- [(4-trifluoromethyl-phenoxy) methyl] piperidinedihydrochloride); SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidinehydrochloride); NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole); CNS 1237 (N-acenaphthyl-N'-4-methoxynaphth-1-yl guanidine), riluzole and analogs thereof may also exhibit specificity for the $Ca_V2.2e[37a]$.

The invention facilitates the development of analogs of these and other calcium channel inhibitors by providing a specific target molecule that is selectively expressed in nociceptive inhibitors. Useful assays are described below and in the Examples in greater detail. The assays of the invention permit the selection of inhibitors, including those mentioned above, analogs and derivatives of those mentioned above, and other molecules that are selective or preferential antagonists (or agonists) of $Ca_V2.2e[37a]$.

Calcium influx assays can be performed to screen and/or determine whether a $Ca_V2.2e[37a]$ inhibitor has the ability to inhibit $Ca_V2.2e[37a]$ activity, and whether the inhibition is selective or preferential. As used herein, "inhibit" refers to inhibiting by at least 10% voltage gated calcium influx, preferably inhibiting by at least 25% voltage gated calcium influx, and more preferably inhibiting by at least 40% voltage gated calcium influx mediated by $Ca_V2.2e[37a]$ as measured by any of the methods well known in the art. An exemplary assay of voltage gated calcium influx is described below in the Examples. By "selective inhibition" is meant that the inhibitor inhibits voltage gated calcium influx mediated by $Ca_V2.2e[37a]$ but does not significantly inhibit voltage gated calcium influx mediated by the

$Ca_V2.2e[37b]$ alternatively spliced subunit. By "preferential inhibition" is meant that the inhibitor inhibits voltage gated calcium influx mediated by $Ca_V2.2e[37a]$ by at least about 5% more that voltage gated calcium influx mediated by the $Ca_V2.2e[37b]$ alternatively spliced subunit. Preferably, the preferential inhibition is at least about 10% more for $Ca_V2.2e[37a]$, more preferably at least about 20% more for $Ca_V2.2e[37a]$, still more preferably at least about 30% more for $Ca_V2.2e[37a]$, yet more preferably at least about 40% more for $Ca_V2.2e[37a]$, and most preferably at least about 50% more for $Ca_V2.2e[37a]$. Greater differences in inhibition of $Ca_V2.2e[37a]$ than $Ca_V2.2e[37b]$ is contemplated, from 51% all the way up to about 99%, at which point the inhibition may be considered selective.

Inhibitors may selectively or preferentially inhibit $Ca_V2.2e[37a]$ based on the state of depolarization of the membrane with which the $Ca_V2.2e[37a]$ is associated. It is well known that certain compounds preferentially bind to voltage-gated calcium channels at particular voltages. For example, dihydropyridine compounds preferentially bind to L-type voltage-gated calcium channels when the membrane is depolarized. Bean (*Proc. Nat'l. Acad. Sci.* 81:6388, 1984) described the binding of nitrendipine to cardiac L-type channels only when the membrane is depolarized. Similar results have been found for nimodipine action in sensory neurons (McCarthy & TanPiengco, *J. Neurosci.* 12:2225, 1992).

Activators of $Ca_V2.2e[37a]$ activity also are enhanced by the invention. Activators may be identified and/or tested using methods described above for inhibitors.

In screening for modulators of $Ca_V2.2e[37a]$, including inhibitors and activators (i.e. antagonists and agonists), it is preferred that compounds (e.g., libraries of potential channel inhibitors) are tested for modulation of $Ca_V2.2e[37a]$ activity at a variety of voltages which cause partial or complete membrane depolarization, or hyperpolarization. These assays are conducted according to standard procedures of testing calcium channel function (e.g. patch clamping, fluorescent Ca^{2+} influx assays) which require no more than routine experimentation. Using such methods, modulators of $Ca_V2.2e[37a]$ activity which are active at particular voltages (e.g., complete membrane depolarization) can be identified. Such compounds are useful for selectively modulating calcium channel activity in conditions which may display voltage dependence. For example, following a stroke membranes are depolarized and such compounds may be active in selectively blocking calcium channel activity for treatment of stroke. Other uses will be apparent to one of ordinary skill in the art.

The invention further provides efficient methods of identifying pharmacological

agents or lead compounds for agents useful in the treatment of conditions associated with voltage gated cell calcium influx mediated by calcium channels containing $Ca_V2.2e[37a]$ subunits and the compounds and agents so identified. Generally, the screening methods involve assaying for compounds which inhibit or enhance voltage gated calcium influx through human N-type calcium channels. Such methods are adaptable to automated, high throughput screening of compounds. Examples of such methods are described in US patent 5,429,921.

A variety of assays for pharmacological agents are provided, including, labeled *in vitro* protein binding assays, Ca^{2+} influx assays, etc. For example, protein binding screens are used to rapidly examine the binding of candidate pharmacological agents to a $Ca_V2.2e[37a]$ polypeptide. The candidate pharmacological agents can be derived from, for example, combinatorial peptide libraries, combinatorial chemical compound libraries, and natural products libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of calcium influx involves contacting a neuronal cell that expresses $Ca_V2.2e[37a]$ polypeptides in conjunction with other polypeptides that constitute functional N-type calcium channels with a candidate pharmacological agent under conditions whereby the influx of calcium can be stimulated by application of a voltage to the test system, i.e., by membrane depolarization. Specific conditions are well known in the art and are described in Lin et al., *Neuron* 18:153-166, 1997, and in US patent 5,429,921. A reduction in the voltage gated calcium influx in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent reduces the induction of calcium influx in cells expressing the $Ca_V2.2e[37a]$ subunit in response to the voltage stimulus. An increase in the voltage gated calcium influx in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent increases the induction of calcium influx in cells expressing the $Ca_V2.2e[37a]$ subunit in response to the voltage stimulus. Methods for determining changes in the intracellular calcium concentration are known in the art and are described elsewhere herein.

$Ca_V2.2e[37a]$ molecules used in the methods of the invention can be added to an assay mixture as an isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a $Ca_V2.2e[37a]$ polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the $Ca_V2.2e[37a]$ molecule as a preloaded polypeptide or as a

nucleic acid (e.g. a cell transfected with an expression vector containing a nucleic acid that encodes a $Ca_V2.2e[37a]$ polypeptide). In the assays described herein, the $Ca_V2.2e[37a]$ polypeptide can be produced recombinantly, or isolated from biological extracts, but preferably is synthesized *in vitro*. $Ca_V2.2e[37a]$ polypeptides encompass chimeric proteins comprising a fusion of a $Ca_V2.2e[37a]$ polypeptide with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, or enhancing stability of the $Ca_V2.2e[37a]$ polypeptide under assay conditions. A polypeptide fused to a $Ca_V2.2e[37a]$ polypeptide or fragment thereof may also provide means of readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

In certain embodiments, the assay includes a control to account for binding and/or modulation of activity of $Ca_V2.2e[37b]$ molecules. As above, this can be prepared by expressing a $Ca_V2.2e[37b]$ nucleic acid in cells and screening the library of compounds with the cells in parallel with the screening using the $Ca_V2.2e[37a]$-expressing cells. The $Ca_V2.2e[37b]$ control cells also can be used to test only positive hit compounds, i.e., those that modulate $Ca_V2.2e[37a]$ activity, for their effect on $Ca_V2.2e[37b]$ activity. In some instances, the cells can express both $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$ molecules.

For the cell-based assay described herein, preferred cell types are neurons and other cells that permit expression of N-type calcium channels including $Ca_V2.2e[37a]$ molecules, including oocytes as shown in the Examples.

The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500. Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also

can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids having non-natural bonds or subunits are also contemplated. Thus, antisense and siRNA molecules can be tested for inhibition of $Ca_V2.2e[37a]$ expression by these assays and other standard assays of nucleic acid expression, such as PCR. Utilizing the cell-based assays described above allows the identification of antisense and siRNA molecules that inhibit function of $Ca_V2.2e[37a]$.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

Candidate agents can be selected randomly or can be based on existing compounds which bind to and/or modulate the function of N-type calcium channels. For example, compounds which are known to inhibit N-type calcium channels include fluspirilene, ziconotide (SNX-111), the ω-conotoxin peptides, as well as small organic molecule calcium channel inhibitors, such as fluspirilene, NNC 09-0026(-)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethyl-phenoxy) methyl] piperidinedihydrochloride); SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidinehydrochloride); NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole); CNS 1237 (N-acenaphthyl-N'-4-methoxynaphth-1-yl guanidine) and riluzole. Therefore, a source of candidate agents are libraries of molecules based on the foregoing N-type calcium channel inhibitors, in which the structure of the inhibitor is changed at one or more positions of the molecule to contain more or fewer chemical moieties or different chemical moieties. The

structural changes made to the molecules in creating the libraries of analog inhibitors can be directed, random, or a combination of both directed and random substitutions and/or additions. One of ordinary skill in the art in the preparation of combinatorial libraries can readily prepare such libraries based on the existing N-type calcium channel inhibitors.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, N-type calcium channels containing the $Ca_V2.2e[37a]$ subunit transduces a control amount of voltage gated calcium influx. For determining the binding of a candidate pharmaceutical agent to a $Ca_V2.2e[37a]$ molecule, the mixture is incubated under conditions which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

After incubation, the level of voltage gated calcium influx or the level of specific binding between the $Ca_V2.2e[37a]$ polypeptide and the candidate pharmaceutical agent is detected by any convenient method available to the user. For cell free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximize signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead,

particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as a calcium influx assay. The calcium influx resulting from voltage stimulus of a N-type calcium channel containing the $Ca_V2.2e$[37a] polypeptide typically alters a directly or indirectly detectable product, e.g., a calcium sensitive molecule such as fura-2-AM. For cell free binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc). or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horseradish peroxidase, etc.). The label may be bound to a $Ca_V2.2e$[37a] polypeptide or the candidate pharmacological agent.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

Thus the invention provides numerous assays using $Ca_V2.2e$[37a] molecules to identify modulators, preferably inhibitors, of $Ca_V2.2e$[37a] function. In one embodiment the $Ca_V2.2e$[37a] inhibitor is an antisense oligonucleotide or siRNA molecule that selectively binds to a $Ca_V2.2e$[37a] nucleic acid molecule, to reduce the expression of $Ca_V2.2e$[37a] in a cell. This is desirable in virtually any medical condition wherein a reduction of $Ca_V2.2e$[37a] activity is desirable, e.g., pain.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of

that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence.

As used herein, a "siRNA molecule" is a double stranded RNA molecule (dsRNA) consisting of a sense and an antisense strand, which are complementary (Tuschl, T. et al., 1999, Genes & Dev., 13:3191-3197; Elbashir, S.M. et al., 2001, EMBO J., 20:6877-6888). In one embodiment the last nucleotide at the 3' end of the antisense strand may be any nucleotide and is not required to be complementary to the region of the target gene. The siRNA molecule may be 19-23 nucleotides in length in some embodiments. In other embodiments, the siRNA is longer but forms a hairpin structure of 19-23 nucleotides in length. In still other embodiments, the siRNA is formed in the cell by digestion of double stranded RNA molecule that is longer than 19-23 nucleotides. The siRNA molecule preferably includes an overhang on one or both ends, preferably a 3' overhang, and more preferably a two nucleotide 3' overhang on the sense strand. In another preferred embodiment, the two nucleotide overhang is thymidine-thymidine (TT). The siRNA molecule corresponds to at least a portion of the $Ca_V2.2e$[37a] gene, but preferably a portion that includes or overlaps at least a portion of exon 37. In a preferred embodiment the first nucleotide of the siRNA molecule is a purine. Many variations of siRNA and other double stranded RNA molecules useful for RNAi inhibition of gene expression will be known to one of ordinary skill in the art.

The siRNA molecules can be plasmid-based. In a preferred method, a polypeptide encoding sequence of $Ca_V2.2e$[37a] is amplified using the well known technique of polymerase chain reaction (PCR). The use of the entire polypeptide encoding sequence is not necessary; as is well known in the art, a portion of the polypeptide encoding sequence is sufficient for RNA interference. For example, the PCR fragment can be inserted into a vector using routine techniques well known to those of skill in the art. The insert can be placed between two promoters oriented in opposite directions, such that two complementary RNA molecules are produced that hybridize to form the siRNA molecule. Alternatively, the siRNA molecule is synthesized as a single RNA molecule that self-hybridizes to form a siRNA

duplex, preferably with a non-hybridizing sequence that forms a "loop" between the hybridizing sequences. Preferably the nucleotide encoding sequence is part of the coding sequence of $Ca_V2.2e[37a]$, specifically exon 37a to confer specificity. The siRNA can be expressed from a vector introduced into cells.

Vectors comprising any of the nucleotide coding sequences of the invention are provided for production of siRNA, preferably vectors that include promoters active in mammalian cells. Non-limiting examples of vectors are the pSUPER RNAi series of vectors (Brummelkamp, T.R. et al., 2002, Science, 296:550-553; available commercially from OligoEngine, Inc., Seattle, WA). In one embodiment a partially self-complementary nucleotide coding sequence can be inserted into the mammalian vector using restriction sites, creating a stem-loop structure. In a preferred embodiment, the mammalian vector comprises the polymerase-III H1-RNA gene promoter. The polymerase-III H1-RNA promoter produces a RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines (T5) in a row. The cleavage of the transcript at the termination site occurs after the second uridine and yields a transcript resembling the ends of synthetic siRNAs containing two 3' overhanging T or U nucleotides. Other promoters useful in siRNA vectors will be known to one of ordinary skill in the art.

Vector systems for siRNA expression in mammalian cells include pSUPER RNAi system described above. Other examples include but are not limited to pSUPER.neo, pSUPER.neo+gfp and pSUPER.puro (OligoEngine, Inc.); BLOCK-iT T7-TOPO linker, pcDNA1.2/V5-GW/lacZ, pENTR/U6, pLenti6-GW/U6-laminshrna and pLenti6/BLOCK-iT-DEST (Invitrogen). These vectors and others are available from commercial suppliers.

It is preferred that the antisense oligonucleotide or siRNA molecule be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon sequences encoding $Ca_V2.2e[37a]$ polypeptides, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense or siRNA molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been

used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. For siRNA molecules, it is preferred that the molecules be 21-23 nucleotides in length, with a 3' 2 nucleotide overhang, although shorter and longer molecules and molecules without overhangs are also contemplated as usefull in accordance with the inveniton.

In accordance with the identification of $Ca_V2.2e$[37a] as selectively or preferentially expressed in nociceptive neurons, oligonucleotides useful in selectively reducing expression of $Ca_V2.2e$[37a] are chosen to be complementary to the region of the mRNA transcripts that encode exon 37a for the preparation of antisense or siRNA molecules. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which polypeptides are not expected to bind. Other methods for selecting preferred siRNA sequences are known to those of skill in the art (e.g., the "siRNA Selection Program" of the Whitehead Institute for Biomedical Research (2003)).

In one set of embodiments, the antisense oligonucleotides or siRNA molecules of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors, including *in situ*.

In preferred embodiments, however, the antisense oligonucleotides or siRNA molecules of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred

synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding $Ca_V2.2e$[37a] polypeptides, together with pharmaceutically acceptable carriers.

Antisense oligonucleotides or siRNA molecules may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the antisense oligonucleotides or siRNA molecules in combination with any standard pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the antisense oligonucleotides or siRNA molecules in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The characteristics of the carrier will depend on the route of administration. Pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

Agents which bind $Ca_V2.2e$[37a] also include binding peptides which bind to the $Ca_V2.2e$[37a] and complexes containing the $Ca_V2.2e$[37a]. When the binding polypeptides are inhibitors, the polypeptides bind to and inhibit the activity of $Ca_V2.2e$[37a]. To determine whether a $Ca_V2.2e$[37a] binding peptide binds to $Ca_V2.2e$[37a], any known binding assay may be employed. For example, the peptide may be immobilized on a surface and then contacted with a labeled $Ca_V2.2e$[37a] polypeptide. The amount of $Ca_V2.2e$[37a] which interacts with the $Ca_V2.2e$[37a] binding peptide or the amount which does not bind to the $Ca_V2.2e$[37a] binding peptide may then be quantitated to determine whether the $Ca_V2.2e$[37a]

binding peptide binds to $Ca_V2.2e[37a]$. Further, the binding of a $Ca_V2.2e[37a]$ polypeptide and a $Ca_V2.2e[37b]$ peptide can be compared to determine if the binding peptide binds selectively or preferentially.

The $Ca_V2.2e[37a]$ binding peptides include peptides of numerous size and type that bind selectively or preferentially to $Ca_V2.2e[37a]$ polypeptides, and complexes of both $Ca_V2.2e[37a]$ polypeptides and their binding partners. These peptides may be derived from a variety of sources. For example, $Ca_V2.2e[37a]$ binding peptides may include known N-type calcium channel inhibitors such as the ω-conotoxin peptides or modified variants of the foregoing peptides. Binding peptides for $Ca_V2.2e[37a]$ polypeptides also can be identified by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the $Ca_V2.2e[37a]$ polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the $Ca_V2.2e[37a]$ polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the $Ca_V2.2e[37a]$ polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the $Ca_V2.2e[37a]$ polypeptides. Thus, the $Ca_V2.2e[37a]$ polypeptides of the invention, or a fragment thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the $Ca_V2.2e[37a]$ polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of $Ca_V2.2e[37a]$ and for other purposes that will be apparent to those of ordinary skill in the art.

Peptides may easily be synthesized or produced by recombinant means by those of skill in the art. Using routine procedures known to those of ordinary skill in the art, one can determine whether a peptide which binds to $Ca_V2.2e[37a]$ is useful according to the invention by determining whether the peptide is one which inhibits the activity of $Ca_V2.2e[37a]$ such as in a voltage gated calcium influx assay, as discussed herein.

The $Ca_V2.2e[37a]$ binding peptide agent may also be an antibody or a functionally active antibody fragment. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining $Ca_V2.2e[37a]$ binding ability (antigen binding fragments). Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also well-known active fragments such as $F(ab')_2$ and Fab and Fv (including single chain antibodies).

An antibody refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1 , CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

The term "antigen-binding fragment" of an antibody as used herein, refers to one or more portions of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term

"antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546) which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding fragment" of an antibody. These antibody fragments are obtained using conventional procedures, such as proteolytic fragmentation procedures, as described in J. Goding, Monoclonal Antibodies: Principles and Practice, pp 98-118 (N.Y. Academic Press 1983), which is hereby incorporated by reference as well as by other techniques known to those with skill in the art. The fragments are screened for utility in the same manner as are intact antibodies.

An "isolated antibody", as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (e.g., a population of isolated antibodies that specifically binds to $Ca_V2.2e[37a]$, is substantially free of antibodies that specifically bind antigens other than a $Ca_V2.2e[37a]$ molecule). An isolated antibody that specifically binds to an epitope, isoform or variant of $Ca_V2.2e[37a]$ may, however, have cross-reactivity to other related antigens, e.g., from other species (e.g., species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. As used herein, "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds $Ca_V2.2e[37a]$ or a closely-related antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (e.g., BSA, casein) or $Ca_V2.2e[37b]$.

The isolated antibodies of the invention encompass various antibody isotypes, such as IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, IgE. As used herein, "isotype" refers to the antibody class (e.g. IgM or IgG1) that is encoded by heavy chain constant region genes.

The antibodies can be full length or can include only an antigen-binding fragment such as the antibody constant and/or variable domain of IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD or IgE or could consist of a Fab fragment, a F(ab')2 fragment, and a Fv fragment.

As used herein, antibodies also include single chain antibodies (e.g., scFvs). In some embodiments, the single chain antibodies are disulfide-free antibodies having mutations e.g., in disulfide bond forming cysteine residues. The antibodies may be prepared by starting with any of a variety of methods, including administering $Ca_V2.2e$[37a]protein, fragments of $Ca_V2.2e$[37a] protein that include SEQ ID NO:45, cells expressing the protein or fragments thereof and the like to an animal to induce polyclonal antibodies. Such antibodies or antigen-binding fragments thereof may be used in the preparation of scFvs and disulfide-free variants thereof. The antibodies or antigen-binding fragments thereof may be used for example to modulate the activity of a target protein (e.g. $Ca_V2.2e$[37a]).

Various forms of the antibody polypeptide or encoding nucleic acid can be administered and delivered to a mammalian cell (e.g., by virus or liposomes, or by any other suitable methods known in the art). The method of delivery can be modified to target certain cells, and in particular, cell surface receptor molecules or antigens present on specific cell types. Methods of targeting cells to deliver nucleic acid constructs, for intracellular expression of the antibodies, are known in the art. The antibody polypeptide sequence can also be delivered into cells by expressing a recombinant protein fused with peptide carrier molecules. These carrier molecules, which are also referred to herein as protein transduction domains (PTDs), and methods for their use, are known in the art. Examples of PTDs, though not intended to be limiting, are tat, antennapedia, and synthetic poly-arginine. These delivery methods are known to those of skill in the art and are described in US patent 6,080,724, and US patent 5,783,662, the entire contents of which are hereby incorporated by reference.

The antibodies of the present invention can be polyclonal, monoclonal, or a mixture of polyclonal and monoclonal antibodies. The antibodies can be produced by a variety of techniques well known in the art. Procedures for raising polyclonal antibodies are well known and are disclosed for example in E. Harlow, et. al., editors, Antibodies: A Laboratory Manual (1988), which is hereby incorporated by reference.

Monoclonal antibody production may be effected by techniques which are also well known in the art. The term "monoclonal antibody," as used herein, refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody displays a

single binding specificity and affinity for a particular epitope. The process of monoclonal antibody production involves obtaining immune somatic cells with the potential for producing antibody, in particular B lymphocytes, which have been previously immunized with the antigen of interest either *in vivo* or *in vitro* and that are suitable for fusion with a B-cell myeloma line.

Mammalian lymphocytes typically are immunized by *in vivo* immunization of the animal (e.g., a mouse) with the desired protein or polypeptide, e.g., with a $Ca_V2.2e[37a]$ polypeptide. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Once immunized, animals can be used as a source of antibody-producing lymphocytes. Following the last antigen boost, the animals are sacrificed and spleen cells removed. See; Goding (in Monoclonal Antibodies: Principles and Practice, 2d ed., pp. 60-61, Orlando, Fla., Academic Press, 1986).

The antibody-secreting lymphocytes are then fused with (mouse) B cell myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured, and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, and grown either *in vivo* or *in vitro* to produce large quantities of antibody. A description of the theoretical basis and practical methodology of fusing such cells is set forth in Kohler and Milstein, Nature 256:495 (1975), which is hereby incorporated by reference.

Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of the desired hybridomas. Examples of such myeloma cell lines that may be used for the production of fused cell lines include P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4.1, Sp2/0-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7, S194/5XX0 Bul, all derived from mice; R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210 derived from rats and U-266, GM1500-GRG2, LICR-LON-HMy2, UC729-6, all derived from humans (Goding, in Monoclonal Antibodies: Principles and Practice, 2d ed., pp. 65-66, Orlando, Fla., Academic Press, 1986; Campbell, in Monoclonal Antibody Technology, Laboratory Techniques in Biochemistry and Molecular Biology Vol. 13, Burden and Von Knippenberg, eds. pp. 75-83, Amsterdam, Elseview, 1984).

Fusion with mammalian myeloma cells or other fusion partners capable of replicating

indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol ("PEG") or other fusing agents (See Milstein and Kohler, Eur. J. Immunol. 6:511 (1976), which is hereby incorporated by reference).

In other embodiments, the antibodies can be recombinant antibodies. The term "recombinant antibody", as used herein, is intended to include antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from an animal (e.g., a mouse) that is transgenic for another species' immunoglobulin genes, antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial antibody library, or antibodies prepared, expressed, created or isolated by any other means that involves splicing of immunoglobulin gene sequences to other DNA sequences.

In yet other embodiments, the antibodies can be chimeric or humanized antibodies. As used herein, the term "chimeric antibody" refers to an antibody, that combines the murine variable or hypervariable regions with the human constant region or constant and variable framework regions. As used herein, the term "humanized antibody" refers to an antibody that retains only the antigen-binding CDRs from the parent antibody in association with human framework regions (see, Waldmann, 1991, Science 252:1657). Such chimeric or humanized antibodies retaining binding specificity of the murine antibody are expected to have reduced immunogenicity when administered in vivo for diagnostic, prophylactic or therapeutic applications according to the invention.

In certain embodiments, the antibodies are human antibodies. The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse have been grafted onto human framework sequences (referred to herein as "humanized antibodies"). Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. patents 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of

which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals results in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies are prepared according to standard hybridoma technology. These monoclonal antibodies have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (HAMA) responses when administered to humans. In particular, mouse strains that have human immunoglobulin genes inserted in the genome (and which cannot produce mouse immunoglobulins) are preferred. Such mice produce fully human immunoglobulin molecules in response to immunization.

According to the invention $Ca_V2.2e[37a]$ inhibitors also include "dominant negative" polypeptides. A dominant negative polypeptide is an inactive variant of a polypeptide, which, by interacting with the cellular machinery, displaces an active polypeptide from its interaction with the cellular machinery or competes with the active polypeptide, thereby reducing the effect of the active polypeptide. For example, a dominant negative $Ca_V2.2e[37a]$ subunit of an active complex (e.g. N-type calcium channel) can interact with the complex but prevent the activity of the complex (e.g., voltage gated calcium influx).

Alternatively, non-human transgenic animal can be prepared using the $Ca_V2.2e[37a]$ nucleic acid described herein. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell, or which contain nucleic acid molecules that are expressed in a cell transiently. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. A variety of methods for the production of transgenic animals are well known in the art. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the affects of a variety of drugs and treatment methods on the disease, and thus serve as genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of $Ca_V2.2e[37a]$ transgenic animals as models for the study of disorders

involving voltage gated calcium influx in nociceptive neurons.

Inactivation or replacement of exon 37a of the endogenous $Ca_V2.2$ subunit gene can be achieved, for example, by a homologous recombination system using embryonic stem cells. The resultant transgenic non-human mammals (preferably primates) having a knockout $Ca_V2.2e[37a]$ characteristic may be used as a model for the effects of compounds that selectively or preferentially inhibit $Ca_V2.2e[37a]$ activity. A similar effect can be achieved by testing the antisense or siRNA molecules described herein in order to prepare "knockdown" animals in which expression of $Ca_V2.2e[37a]$ is reduced or eliminated. Such animals also can be used to assess the effects of antisense or siRNA therapies.

The compositions of the invention are also useful for therapeutic purposes. Accordingly the invention encompasses a method for modulating (reducing or increasing) $Ca_V2.2e[37a]$ activity in a mammalian cell. In preferred embodiments, the method involves contacting the mammalian cell with an amount of a $Ca_V2.2e[37a]$ inhibitor effective to inhibit voltage gated calcium influx in the mammalian cell. Such methods are useful *in vitro* for altering voltage gated calcium influx for the purpose of, for example, elucidating the mechanisms involved in pain, e.g., neuropathic pain, and for restoring normal voltage gated calcium influx in a cell having a defective (excessively active) $Ca_V2.2e[37a]$. *In vivo*, such methods are useful, for example, for reducing N-type voltage gated calcium influx, e.g., to treat pain, e.g., neuropathic pain, or any condition in which $Ca_V2.2e[37a]$ activity is elevated.

An amount of a $Ca_V2.2e[37a]$ inhibitor which is effective to inhibit voltage gated calcium influx in the mammalian cell is an amount which is sufficient to reduce voltage gated calcium influx by at least 10%, preferably at least 20%, more preferably 30% and still more preferably 40%. An amount of a $Ca_V2.2e[37a]$ which is effective to increase voltage gated calcium influx in the mammalian cell is an amount which is sufficient to increase voltage gated calcium influx by at least 10%, preferably at least 20%, more preferably 30% and still more preferably 40%. Such alterations in voltage gated calcium influx can be measured by the assays described herein.

As described above with respect to inhibitors, modulators of $Ca_V2.2e[37a]$ may selectively or preferentially inhibit or increase $Ca_V2.2e[37a]$ function based on the state of depolarization of the membrane with which the $Ca_V2.2e[37a]$ polypeptide is associated. Therefore, in screening for modulators of $Ca_V2.2e[37a]$, it is preferred that compounds (e.g. synthetic combinatorial libraries, natural products, peptide libraries, etc.) are tested for

modulation of $Ca_V2.2e[37a]$ activity at a variety of voltages which cause partial or complete membrane depolarization, or hyperpolarization. These assays are conducted according to standard procedures of testing calcium channel function (e.g. patch clamping, fluorescent Ca^{2+} influx assays) which require no more than routine experimentation. Using such methods, modulators of $Ca_V2.2e[37a]$ activity which are active at particular voltages (e.g. complete membrane depolarization) can be identified. Such compounds are useful for selectively modulating calcium channel activity in conditions which may display voltage dependence.

The invention also encompasses a method for increasing $Ca_V2.2e[37a]$ expression in a cell. It is desirable to increase $Ca_V2.2e[37a]$ in a cell, for example, to prepare cells useful in the assays described herein. The amount of $Ca_V2.2e[37a]$ can be increased in such cell by contacting the cell with a $Ca_V2.2e[37a]$ nucleic acid or a $Ca_V2.2e[37a]$ polypeptide of the invention in an amount effective to increase voltage gated calcium influx in the cell. An increase in $Ca_V2.2e[37a]$ activity can be measured by the assays described herein, e.g., assays of calcium influx.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response. In the case of treating a condition characterized by aberrant voltage gated calcium influx, the desired response is reducing or increasing calcium influx to a level which is within a normal range. Preferably, the change in calcium influx produces a detectable reduction in a physiological function related to the condition, e.g., a reduction in pain. The responses can be monitored by routine methods. In the case of a condition where an increase in voltage gated calcium influx is desired, an effective amount is that amount necessary to increase said influx in the target tissue. The converse is the case when a reduction in influx is desired. When administered for treatment of pain conditions, the effectiveness of inhibitors of $Ca_V2.2e[37a]$ can be assessed directly by querying the patient regarding,e.g., the extent and duration of pain relief.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. It is preferred generally that a maximum dose be used, that is, the highest

safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

Generally, doses of active compounds would be from about 0.01 ng/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50 μg - 500 mg/kg will be suitable and in one or several administrations per day. Lower doses can result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compound, although fewer doses typically will be given when compounds are prepared as slow release or sustained release medications.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

The $Ca_V2.2e[37a]$ inhibitors or $Ca_V2.2e[37a]$ nucleic acids and polypeptides useful according to the invention may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular compound selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intrathecal, intramuscular, or infusion.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the $Ca_V2.2e[37a]$ inhibitor or $Ca_V2.2e[37a]$ nucleic acids and polypeptides, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or

di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intrathecal, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the active compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. Use of a long-term sustained release implant may be desirable. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples

Example 1: Analysis of N-type calcium current and $Ca_V2.2a1$ splice variants in nociceptive neurons

N-type calcium channels are important for pain transduction. The N-type $Ca_V2.2$ $\alpha 1$ gene contains a number of exons that are subject to tissue-specific alternative splicing (Lipscombe et al., 2002). Dorsal root ganglia (DRG) neurons express multiple $Ca_V2.2$ $\alpha 1$ splice isoforms (Bell et al., 2001). Here we report that exon 37a (e37a), located in the C-terminus of $Ca_V2.2$ $\alpha 1$, is expressed in a subset of nociceptive neurons. E37a encodes 32 amino acids in the C-terminus, is expressed in a mutually exclusive manner with e37b, and disrupts a putative EF-hand motif. The cell-specific expression of e37a prompted us to compare the functional properties of N-type calcium channel currents in dorsal root ganglia. Nociceptive neurons express signature markers such as the non-specific cation channel VR-1 (Caterina et al., 1997). DRG neurons were therefore operationally defined as nociceptive if

they responded to the VR-1 receptor ligand capsaicin. We found that capsaicin-responsive neurons containing e37a have a statistically greater peak N-type current density compared to capsaicin-responsive neurons that lack e37a.

Materials and Methods

Calcium current recordings

Acutely dissociated DRG neurons were placed into the recording chamber containing Tyrodes solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, and 15 mM Dextrose; the pH was adjusted to 7.2 with NaOH). Patch electrodes were fabricated from borosilicate glass and polished to a resistance of 1-2 MΩ. The internal solution for the perforated patch recordings consisted of 135 mM CsCl, 10 mM HEPES, 1 mM EGTA, 1 mM EDTA, 4 mM $MgCl_2$ 1.2 pg/ml amphotericin and adjusted to a pH of 7.2 with TEA-OH. Seals were formed in Tyrodes solution. The series resistance decreased over a 20 min period and deemed acceptable when it fell below 12 MΩ. The bath solution was then replaced with 135 mM TEA-Cl, 2 mM $CaCl_2$, 10 mM HEPES, 100 nM TTX and adjusted to a pH of 7.2 with TEA-OH. Whole cell calcium currents were recorded under constant bath perfusion. A current voltage protocol from a holding potential of –80 mV with 50ms test pulses was then delivered. ω-Ctx GVIA (3 μM) was then applied via a second pipette using positive pressure. Test pulses at 20 second intervals were applied during toxin application. When toxin block was maximal (three test pulses generating equal sized currents) a second current voltage protocol was delivered. Finally the bath solution was exchanged with Tyrodes and the cell was held at –60 mV. 2 μM capsaicin in Tyrodes was then exposed to the cell. The entire cell was then harvested and stored in first-strand buffer at –80 until needed. After each application of ω-Ctx GVIA, the recording chamber was soaked in 2% SDS and 10 mM DTT for 15 min to remove any residual ω-Ctx GVIA. Data acquisition was performed using pClamp V8.1 software and the Axopatch 200A (Axon Instruments). For recordings of calcium current data was filtered at 2kHz and sampled at 20kHz. The series resistance was compensated by 70-90% with a 10ms lag. Leak correction was performed with a p/-4 protocol. For recordings of VR-1 current data was filtered at 2kHz and sampled at 2kHz. No leak subtraction or series resistance compensation was performed. All recordings were obtained at room temperature.

Capsaicin screening

Viable neurons were identified using whole cell voltage clamp recordings based on the presence of robust voltage-gated sodium and potassium currents. Neurons were then exposed to 2 μM capsaicin (Sigma). After exposure, the bath was washed and the cell allowed to recover. Capsaicin-responsive cells had to recover after wash to be included. Capsaicin-non-responsive neurons were expose at least twice to capsaicin before the cell was collected and counted as capsaicin-non-responsive. Coverslips in the study were limited to two exposures of capsaicin to avoid desensitization. 1-2 MΩ microelectrodes were filled with an internal solution consisting of 90 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM HEPES, 5 mM EGTA and the pH was adjusted to 7.4 with KOH. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, and 15 mM Dextrose. The pH was adjusted to 7.2 with NaOH. At the conclusion of the recording the entire cell was harvested via the recording pipette by negative pressure and stored in first-strand buffer at –80°C until needed. Data acquisition was performed using PClamp V8.1 software and Axopatch 200A (Axon Instruments). Data was filtered at 2kHz and sampled at 2kHz. No leak correction or series resistance compensation was performed. The recordings were obtained at room temperature.

Single cell RT-PCR protocol

The electrode tip and its contents (~2 μl) were broken into a thin-walled PCR tube containing water and First-strand buffer. cDNA was synthesized from single cells according the Superscript II protocol with minor modifications. cDNA from single cells was then used as a template for subsequent PCR reactions with primers for GAPDH (0.1 of cDNA) and $Ca_V2.2\alpha1$ e37a or e37b (~0.5 of cDNA). Products were then run out on 2% standard agarose and stained with EthBr. Competitive PCR templates were spiked into PCR reactions for either e37a and e37b. For 1ng DRG tissue (~10 cells) competitor dilutions of 10^{-18} -10^{-22} and for single cell cDNA 10-20 were used. Products were then run out on 4% Metaphor agarose gels and stained with EthBr. Band intensities were directly quantified from gels using a digital analysis system (Alpha Innotech).

Results

Fig. 1 shows RT-PCR analysis of e37a and e37b splice variants. RT-PCR analysis of e37a and e37b splice variants of Cav2.2 from rat dorsal root ganglia showed that $Ca_V2.2$ containing exons 37a or e37b were expressed in rat DRG tissue (P5).

Fig. 2 shows RT-PCR analysis of e37a and e37b in single DRG neurons. The PCR primer pairs were exon–specific for e37a and e37b. The predicted PCR product was 108 bp for both e37a and e37b. Fig. 2A, Representative gel for scRT-PCR for e37a, e37b and GAPDH in the same neuron. Fig. 2B, Diagrams of the PCR primer locations for each site.

Fig. 3 shows single cell RT-PCR (scRT-PCR) analysis of $Ca_V2.2$ e37a and e37b in capsaicin-responsive and capsaicin-non-responsive neurons. Fig 3A, Representative gel for scRT-PCR for e37a and GAPDH. Fig 3B, Representative gel for scRT-PCR for e37b and GAPDH. Fig 3C, Bar graph summary of e37a and e37b results.

Exon 37a was expressed only in a subset of neurons. e37a PCR products were detected in 55% of capsaicin-responsive neurons (32 of 58) but in only 19% (5 of 27) of non-responsive neurons. This result suggests that exon 37a is expressed in a subset of nociceptive neurons in the DRG.

Fig. 4 shows the experimental protocol for certain experiments. DRG neurons from P5-8 rats were dissociated and immediately plated on glass coverslips. Fig. 4A, Whole cell voltage-clamp recordings of calcium currents were performed using the perforated patch technique. Current-voltage curves were obtained from each cell (holding potential = –80 mV). Fig. 4B, 3 mM ω-Ctx GVIA was then applied via a micropipette and the onset of block assessed by test pulses to peak current every 20 seconds. At maximum block a second I-V curve was obtained. Fig. 4C, Post-toxin currents were digitally subtracted from pre-toxin currents to reveal the toxin-sensitive component of the whole cell calcium current. Fig. 4D, Finally, 2 mM capsaicin was applied and the presence of an inward current of at least 25 pA at a holding potential of -60 mV, defined a capsaicin-responsive neuron. Fig. 4E, Neurons were collected for single cell RT-PCR analysis of e37a and 37b of $Ca_V2.2$, and GAPDH.

Fig. 5 shows whole cell calcium currents in capsaicin-responsive and capsaicin-non-responsive neurons. Fig. 5A, I-V plots of the whole cell calcium current in capsaicin-

responsive (○) and capsaicin-non-responsive (■) DRG neurons. Fig. 5B, Representative low voltage-activated (LVA) and high voltage-activated (HVA) whole cell calcium currents from a capsaicin-non-responsive neuron. Fig. 5C, Same as Fig. 5B from a capsaicin-responsive neuron. Fig. 5D, Summary of peak calcium current density and capacitance of capsaicin-responsive ($V_{½} \sim$ -15 mV) and non-responsive neurons ($V_{½} \sim$ -45 mV and -15 mV for LVA and HVA, respectively. The whole cell capacitance of capsaicin-non-responsive neurons is significantly greater compared to capsaicin-responsive neurons (Students *t* test; $p<0.001$).

Fig. 6 shows that ω-Ctx GVIA-sensitive calcium currents in capsaicin-responsive and capsaicin-non-responsive neurons. Fig. 6A, I-V plots of ω -Ctx GVIA-sensitive calcium current in capsaicin-responsive (○) and capsaicin-non-responsive (■) DRG neurons. $V_{½}$ = ~-15 mV for capsaicin-responsive and $V_{½}$ = ~-45 mV and -15 mV for LVA and HVA currents in capsaicin-non-responsive neurons. Fig. 6B, Representative ω-Ctx GVIA-sensitive calcium currents. Fig. 6C, Summary of peak calcium channel current density, % ω-Ctx GVIA-sensitive calcium current, and capacitance in capsaicin-responsive and non-responsive neurons.

Fig. 7 shows that LVA currents rundown significantly over a 5 minute time period in capsaicin-non-responsive neurons. The presence of a low voltage-activated GVIA-sensitive current in capsaicin-non-responsive cells is surprising (Fig. 6A). GVIA is well known for is selective effects on N-type HVA currents. We therefore tested the hypothesis that the LVA current ran down over the time period of the experiment even in the absence of GVIA. The same voltage protocol used during the toxin application experiments was employed. Only 52 ± 2% of the LVA current (-40 mV) remained after 5 minutes compared to 89 ± 1% of HVA current (-10 mV). The apparent presence of GVIA-sensitive LVA currents in capsaicin-non-responsive neurons in Figure 6 is simply a function of preferential rundown of LVA currents.

Fig. 8 shows ω-Ctx GVIA-sensitive calcium currents in capsaicin-responsive neurons that contain and lack e37a. Fig. 8A, I-V plots of ω-Ctx GVIA-sensitive calcium currents in capsaicin-responsive neurons that contain (○) and lack (■) e37a. Fig. 8B, Representative gel from scRT-PCR for e37a, e37b, and GAPDH in the same neuron. PCR primer locations are shown. Fig. 8C, Summary of GVIA-sensitive peak calcium current density, % ω-Ctx GVIA-

sensitive calcium current, and capacitance in capsaicin-responsive and non-responsive neurons. The peak N-type current density was significantly greater in capsaicin-responsive neurons containing e37a compared to exon 37a-lacking (Students *t* test; $p<0.05$). $V_{½} \sim -15mV$ for both groups.

Fig. 9 shows competitive RT-PCR analysis of e37a and e37b in whole tissue and single neurons. Using different exon–specific PCR primers for e37a and e37b, we were not able to determine relative levels of each exon. To estimate approximate levels of e37a and e37b we used a competitive single cell RT-PCR protocol. Each primers pair generated two PCR products; a 108 bp product from endogenous $Ca_V2.2$ cDNA and a 135 bp product from the competitive template added in known amounts to PCR reactions. Fig. 9A, Representative gel for RT-PCR of whole DRG tissue for e37a and e37b in the presence of serial dilutions of competitive template (10^{-18} -10^{-22} M). Fig. 9B, Representative gel for scRT-PCR for e37a and e37b with the same competitive template dilution (10^{-20} M) from the same neuron.

Summary

Exon 37a of $Ca_V2.2\alpha1$ is restricted to a subset of DRG neurons. 55% of capsaicin-responsive neurons expressed e37a while only 19% of capsaicin-non-responsive neurons e37a.

Analysis of whole cell calcium current revealed that capsaicin-non-responsive neurons express a low voltage-activating current that is less prominent in capsaicin-responsive neurons. The N-type current density was slightly, but not significantly greater in capsaicin-responsive (108 ± 13 pA/pF) compared to non-responsive neurons (77 ± 9 pA/pF)

Capsaicin-responsive neurons that contain e37a have a significantly larger N-type current (108 ± 16 pA/pF) compared to capsaicin-responsive neurons that lack e37a (65 ± 4 pA/pF). Students *t* test; $p<0.05$).

Conclusions

We have demonstrated that $Ca_V2.2\alpha1$ splice variants containing e37a are preferentially expressed in a subset of nociceptive neurons of the dorsal root ganglia.

The functional significance of exon 37a is not yet known, however, capsaicin-responsive neurons that contain e37a have a larger N-type current compared to those lacking e37a.

It is possible that e37a promotes surface expression of $Ca_V2.2$. The greater N current density in nociceptive neurons expressing e37a may have significance specific to the physiology of pain.

Example 2: Cell-specific alternative splicing increases calcium channel current density in the pain pathway

Experimental Procedures

Whole tissue RT-PCR

Total RNA was isolated from various parts of the rat nervous system with Trizol reagent (Invitrogen, Carlsbad, CA). 2 μg of total RNA from each tissue was reverse transcribed with Superscript II (Invitrogen). 10 ng of the first strand cDNA mixture was analyzed using the Advantage 2 PCR system (BD Biosciences). PCR products were cloned and sequenced to confirm their molecular identity. RT-PCR primers for e37a were: rat37a-up, 5'gctgcgtgttgccggattcattat (SEQ ID NO:1); rat37a-dw, 5'ttcattcgaaccaggcgcttgtag (SEQ ID NO:2) (122 bp) and for e37b: rat37b-up, 5'ctgaatacgacccagctgcgtgtg (SEQ ID NO:3); rat37b-dw, 5'ccaggcgcttgtatgcaactcgag (SEQ ID NO:4) (126 bp). PCR was performed using a Robocycler (Stratagene) programmed as follows: 1 cycle of 94°C for 1 min; followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec; followed by a final 7 min extension at 72°C. Control amplifications were performed with cDNAs to ensure the primers were specific for e37a and e37b. RT-PCR products were run on 3% agarose gels pre-stained with ethidium bromide and imaged on a digital analysis system (Alpha Innotech, San Leandro, CA).

Cell isolation

Dorsal root ganglia were harvested from all spinal levels of P5 to P8 rats (Sprague Dawley, Charles River, Wilmington, MA). Ganglia were dissociated in a solution containing supplemented L-15 media with collagenase (20 mg/ml, Sigma Chemical Co., St. Louis, MO) and dispase (96 mg/ml, Sigma). Enzymatic digestion was carried out at 37°C for ~45 min and cells triturated periodically with a fire polished pipette to facilitate dissociation. Neurons were washed twice in 1X PBS (Gibco Invitrogen) and plated on poly-D-lysine (Sigma) coated covers slips in DMEM (Gibco) containing 10 % FBS (Gibco) and NGF (1 ng/ml, Sigma). Neurons were maintained at 37°C with 5% CO2 and used within 1 to 6 hrs after isolation.

Capsaicin screening

Viable neurons were identified by the presence of robust voltage-gated sodium and potassium currents during brief depolarizations from a holding potential of -80 mV. Cells were then maintained at a holding potential of -60 mV and challenged with 2 μM capsaicin to identify responsive and non-responsive neurons. Neurons were then washed with capsaicin-free media. For inclusion in the analysis, capsaicin-induced inward currents had to be at least 25 pA and recover after capsaicin removal. Capsaicin induced currents ranged from 25 pA to 4500 pA. Capsaicin-non-responsive neurons were exposed to capsaicin at least twice before the cell was harvested and counted as capsaicin-non-responsive. Neurons were limited to two exposures of capsaicin to avoid desensitization. For capsaicin screening, currents were recorded using 1-2 MΩ microelectrodes filled with an internal solution consisting of 90 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$ 40 mM HEPES, and 5 mM EGTA adjusted to a pH of 7.4 with KOH. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, and 15 mM dextrose, adjusted to a pH of 7.2 with NaOH. At the conclusion of the recording the entire cell was harvested via the recording pipette by negative pressure and stored in first-strand buffer at –80°C until needed. Data acquisition was performed using PClamp V8.1 software and the Axopatch 200A (Axon Instruments, Union City, CA). Data was filtered at 2 kHz (-3dB) and sampled at 2 kHz. No leak correction or series resistance compensation was performed. Recordings were performed at room temperature.

Calcium current recordings

Acutely dissociated DRG neurons were placed into the recording chamber containing bathing solution (see above). Patch electrodes were fire polished to a resistance of 1-2 MΩ. The internal solution for perforated patch recordings consisted of 135 mM CsCl, 10 mM HEPES, 1 mM EGTA, 1 mM EDTA, 4 mM $MgCl_2$ and 1.2 mg/ml amphotericin (Sigma), adjusted to a pH of 7.2 with TEA-OH. Micropipettes were pre-filled with amphotericin-free solution and backfilled with internal solution containing amphotericin. Seals were formed in bathing solution. The series resistance decreased over a 20 min period and was considered acceptable when < 12 MΩ. The bath solution was then replaced with 135 mM TEA-Cl, 2 mM $CaCl_2$, 10 mM HEPES, 100 nM TTX (Sigma), adjusted to a pH of 7.2 with TEA-OH. Whole cell calcium currents were recorded under constant bath perfusion. A current-voltage protocol from a holding potential of -80 mV with 50 ms test pulses was then delivered. ω-Ctx GVIA (3 μM; Calbiochem, San Diego, CA) was applied directly onto the cell via a pipette using positive pressure. Test pulses were applied every 20 seconds to monitor toxin block which was considered saturated when current amplitudes were constant over a period of 1 minute. ω-Ctx GVIA was completely removed from the bath and a second current-voltage relationship obtained. Finally, the bath was exchanged with Na-containing solution, the cell voltage clamped to -60 mV, and challenged with 2 μM capsaicin. The entire cell was then harvested and stored in first-strand buffer at -80°C. After each application of ω-Ctx GVIA, the recording chamber was soaked in 2 % SDS and 10 mM DTT for 15 min to remove any residual ω-Ctx GVIA. Data acquisition was performed using pClamp V8.1 software and the Axopatch 200A (Axon Instruments). For recordings calcium currents, data was filtered at 2 kHz (-3dB) and sampled at 20 kHz. The series resistance was compensated by 70-90 % with a 10 μs lag. Leak correction was performed with a P/-4 protocol. All recordings were obtained at room temperature.

The low threshold component in toxin-subtracted currents from capsaicin-nonresponsive cells

A low threshold calcium current was prominent in capsaicin-non-responsive cells (Fig. 15a). This current deactivated with slow kinetics, typical of T-type currents but it was also labile. Current amplitudes declined to 52 ± 2% of initial values over a 5 minute period (measured at -40 mV, n = 3) compared to a decline to 89 ± 1 % for the high threshold current (at –10 mV, n = 3). Relatively rapid run-down of the low threshold current likely explains the

presence of a low threshold current showing up in the ω-Ctx GVIA-sensitive current voltage relationship. It is also possible that other low threshold calcium currents contribute to this component of the current voltage-relationship. For example, $Ca_V1.3$ L-type currents that activate at relatively negative voltages (Lipscombe, 2002). There are reports that the $Ca_V1.3$ L-type current is inhibited by high concentrations of ω-Ctx GVIA (Williams et al., 1992b), however, our studies of neuronal $Ca_V1.3$ L-type channels suggest that they are not ω-Ctx GVIA-sensitive (Xu and Lipscombe, 2001).

Single cell RT-PCR

After harvesting the entire neuron into the recording pipette via negative pressure, the tip of the pipette and its contents (~2 μl) were broken into a thin-walled PCR tube containing water and first-strand buffer. cDNA was synthesized from single cells using Superscript II (Invitrogen) and used as template for subsequent PCR reactions with the Advantage2 PCR system (BD Biosciences Clontech, Palo Alto, CA). One round of PCR was performed using a Robocyler (Stratagene, La Jolla, CA) on single cell cDNA. For all sets of primers the PCR reaction conditions were 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 1 min, 59°C for 1.5 min, 72°C for 1.5 min, and a final 7 min extension at 72°C. Products were then run out on 2 % standard agarose or 4 % Metaphor agarose gels. Band intensities were directly quantified from gels using a digital analysis system (Alpha Innotech). PCR products from single cells were subcloned and sequenced to confirm their identity. Negative controls lacking cDNA were performed and processed in parallel with single cell RT-PCR reactions (1 negative control for every 6 cells). The negative controls included recording pipettes used for whole cell recording. To prevent contamination all PCR reactions were performed in a separate room not previously exposed to $Ca_V2.2$ clones. Internal solutions were RNAse free. RT-PCR primers were located in different exons to avoid amplification of genomic DNA. RT-PCR reagents and enzymes were always divided into aliquots to screen single cells in groups of ~50 neurons with identical stocks of RT-PCR reagents and enzymes. This reduced variability. The PCR primer sequences and their predicted PCR product sizes were as follows: r18a-up, 5'cgcaggttctggagccttagct (SEQ ID NO:5); r18a-dw, 5'ggccattgctgtggacaacctt (SEQ ID NO:6) (227 and 290 bp for .18a and +18a). r24a-up, 5'cattctggacttcattgttgtcagtgga (SEQ ID NO:7); r24a-dw, 5'tcgcaggactctcagagacttgatggta (SEQ ID NO:8) (114 and 126 bp for .24a and +24a). r31a-up, 5'cagagatgcctggaacgtctttgac (SEQ ID NO:9); r31a-dw, 5'ataacaagatgcggatggtgtagcc

(SEQ ID NO:10) (169 and 175 bp for .31a and +31a). r37a-up, 5'agctgcgtgttgccggattcattataagga (SEQ ID NO:11); r37a-dw, 5'gcgcttgtaggccaacctacgagggcagtt (SEQ ID NO:12) (108 bp). r37b-up, 5'agctgcgtgtgggcgcatcagttacaatga(SEQ ID NO:13); r37b-dw, 5'gcgcttgtatgcaactcgagccgggcattt (SEQ ID NO:14) (108 bp). rVR1-up, 5'tcaattcccacacacctcccagttcc (SEQ ID NO:15); rVR1-dw, 5'tttgggtggtctgcttagggaaccag (SEQ ID NO:16) (125 bp). rNaV1.8-up, 5'gcgctggttgtaagggtcagattg (SEQ ID NO:17); rNaV1.8-dw, 5'agccaggcaacaattgcagaaatc (SEQ ID NO:18) (245 bp). rGAPDH-up, 5'cagcaccagcatcaccccattt (SEQ ID NO:19); rGAPDH-dw, 5'caagatggtgaaggtcggtgtgaa (SEQ ID NO:20) (274 bp). Competitive template for e37a, 5'agctgcgtgttgccggattcattataaggataagcgtgctggactctctcgacgcaa atcgaggcccaattggcaaatacagttgcagaacaggaggagccacacaactgccctcgtaggttggcctacaagcgc (SEQ ID NO:21) (135 bp); for e37b, 5'agctgcgtgtgggcgcatcagttgcaatgataagcgtgctggactctctcgacgc aaatcgaggcccaattggcaaat acagttgcagaacaggaggagccacacaaaagcccggctcgagttgcatacaagcgc (SEQ ID NO:22) (135 bp).

Oocyte expression and physiology

Complementary RNAs (cRNAs) were synthesized *in vitro* using the Ambion mMessage machine RNA transcription kit using T-7 primer (Ambion, Austin, TX). 1 µl of each $Ca_V2.2$ cRNA preparation was run out on a denaturing formaldehyde gel along with cRNA standards. Band intensities were directly quantified using a digital analysis system (Quantity-One, Bio- Rad, Hercules, CA) to ensure equal amounts of $Ca_V2.2e$[37a] and $Ca_V2.2e$[37b] cRNAs. Full-length cRNAs were mixed in equimolar ratios, 5 ng of $Ca_V2.2$, 1.6 ng of $Ca_V\beta3$, and 2.6 ng of $Ca_V\alpha_2\delta$ in a total volume of 46 nl, and injected into individual defolliculated oocytes. Four separate rounds of oocyte injections were performed. N-type calcium currents were recorded 4 - 6 days after injection by standard two microelectrode voltage clamp technique (Warner amplifier; OC-725b) and the data collected using pClamp6 software (Axon Instruments, Union City, CA). Microelectrodes were filled with 3M KCl and the resistances of the current and voltage electrodes were 0.3-1.5 MΩ. Data were filtered at 0.5 kHz and sampled at 10 kHz. Currents were recorded in a reduced chloride bathing solution containing: 5 mM $Ba(OH)_2$, 5 mM HEPES, 85 mM TEA-OH, and 2 mM KOH, pH adjusted to 7.4 with methanesulfonic acid. N-type currents ranged between 0.1 µA and 0.6

μA. Leak and capacitive currents were subtracted online using a P/-4 protocol (Pclamp, V6.0; Axon Instruments).

Results

Multiple $Ca_V2.2$ splice isoforms are expressed in dorsal root ganglia

The $Ca_V2.2$ gene is subject to alternative splicing and dorsal root ganglia express multiple splice isoforms (Fig. 10a, b). Cassette exons e18a, e24a, and e31a are either present or not in the final mRNAs. Their expression patterns have been reasonably well characterized at the whole tissue level. The majority of $Ca_V2.2$ mRNA in dorsal root ganglia contains e31a, consistent with preferential expression of this exon in peripheral ganglia. Splice isoforms of $Ca_V2.2$ both containing and lacking e18a and e24a are also present (Fig. 10b). The splicing patterns of e18a, e24a, and e31a of $Ca_V2.2$ at the whole tissue level is consistent with previous analyses by RT-PCR and ribonuclease protection in rat DRG (Lin et al., 1997; Lin et al., 1999; Pan and Lipscombe, 2000) and for equivalent exons 24a and 31a in chick DRG (Lu and Dunlap, 1999). These analyses of whole tissue, however, do not address whether the pattern of alternative splicing varies among individual neurons. Sensitivity to capsaicin is commonly used as a functional marker to identify a subtype of nociceptive neuron (Caterina and Julius, 2001; Caterina et al., 1997; Tominaga et al., 1998). We therefore used whole-cell recording to separate neurons into two groups based on capsaicin-responsiveness, and then analyzed each site of alternative splicing in the same neurons by single cell RT-PCR.

Screening neurons of the dorsal root ganglia by capsaicin-responsiveness

Neurons of the dorsal root ganglia were voltage-clamped at -80 mV, and brief depolarizations applied to establish the presence of robust voltage-gated potassium and sodium currents characteristic of viable neurons. Subsequently, the membrane potential was voltage-clamped to -60 mV and neurons exposed to 2 μM capsaicin (Fig. 11a, b; n = 128 capsaicin-responsive neurons and n = 141 non-responsive neurons). Capsaicin exerts its effects via activation of the vanilloid receptor (VR1). Consistent with this, we detected VR1 mRNA in 89 % of capsaicin-responsive cells (25 of 28) whereas only 13 % of non-responsive cells contained VR1 mRNA (2 of 15) (Fig. 11c, d).

Individual DRG neurons express multiple $Ca_V2.2$ splice isoforms

We analyzed e18a, e24a, and e31a at the single cell level and demonstrated that the pattern of alternative splicing varied among individual neurons. Some cells expressed both isoforms of a particular splice site, while other cells expressed one form (Fig. 12). For example, all neurons expressed $Ca_V2.2$ mRNAs that lacked e18a (Fig. 12a, n = 38 cells). While only a sub-set also expressed $Ca_V2.2$ mRNAs containing e18a. We observed a pattern of alternative splicing similar to this for the e24a site (Fig. 12b, n = 38). However, there was no correlation between the inclusion or exclusion of a particular exon and capsaicin-responsiveness (Fig. 12a, b). We detected e31a in all neurons analyzed regardless of capsaicin responsiveness. $Ca_V2.2$ mRNAs lacking e31a were not detected (Fig 12c; n = 18). This reflects the splicing pattern of e31a observed at the whole tissue level and is consistent with the proposal that the vast majority of $Ca_V2.2$ mRNAs expressed in peripheral ganglia, including DRG, contain e31a (Lin et al., 1997; Lin et al., 1999; Lu and Dunlap, 1999).

Exon 37a is only expressed in dorsal root ganglia

We examined a fourth site of alternative splicing, located in the C-terminus, that had been identified based on analysis of human genomic sequence (Lipscombe, 2003; Lipscombe et al., 2002; Soong et al., 2002). The presence of tandem exons, e37a and e37b in genomic sequence, identical in length (97 nucleotides) and differing by only 14 amino acids is consistent with a mechanism of mutually exclusive splicing (Fig. 13a). However, despite evidence for the presence of these two homologous exons of $Ca_V2.2$ in the public genome sequence of human, rat and mouse, all $Ca_V2.2$ mRNAs analyzed to date contain e37b. No mammalian $Ca_V2.2$ mRNA containing e37a has been reported. We designed two sets of exon-specific PCR primers, because e37a and e37b are indistinguishable on the basis of size. We analyzed their expression patterns in rat, initially at the whole tissue level. As expected, $Ca_V2.2$e[37b] mRNAs were expressed throughout the central nervous system and in both sympathetic and dorsal root ganglia. In sharp contrast, we found expression of e37a highly restricted and only present at significant levels in dorsal root ganglia (Fig. 13b). Our results explain why $Ca_V2.2$e[37a] cDNAs were not isolated in earlier cloning efforts because tissue from dorsal root ganglia was not used (Cahill et al., 2000; Coppola et al., 1994; Dubel et al., 1992; Fujita et al., 1993; Lin et al., 1997; Williams et al., 1992a). Our use of exon-specific primers, however, does not provide information on the relative levels of $Ca_V2.2$e[37a] and $Ca_V2.2$e[37b] mRNAs. The high efficiency of PCR could result in significant product even if

starting levels of $Ca_V2.2e[37a]$ mRNAs are low in number. We therefore employed a competitive RT-PCR protocol and established that $Ca_V2.2e[37a]$ mRNAs are present in whole DRG of P5 and adult animals at levels only ~10-fold lower compared to $Ca_V2.2e[37b]$ (Fig. 13c and 13d). The $Ca_V2.2e[37a]$ splice isoform therefore represents a significant fraction of the total $Ca_V2.2$ mRNA pool in dorsal root ganglia. We next tested the possibility that e37a expression is regulated at the single cell level and perhaps linked to functionally distinct sub-types of sensory neurons.

Preferential expression of exon e37a in a subset of capsaicin-responsive neurons

We separated neurons of the dorsal root ganglia on the basis of capsaicin-responsiveness and analyzed each cell by RT-PCR using e37a and e37b -specific primers. All neurons tested with e37b-specific primers contained e37b (n = 48; Fig. 14a, 14d). This is consistent with the ubiquitous expression pattern of this splice form of $Ca_V2.2$ (Fig. 13b) and also serves as an important control establishing that $Ca_V2.2$ mRNA is expressed in every neuron. By contrast, only a sub-set of neurons expressed e37a. Most significantly, the e37a splice isoform was expressed preferentially in capsaicinresponsive neurons (55 % of capsaicin-responsive compared to 17 % of non-responsive neurons; Fig. 14b, 14d). Capsaicin-sensitivity is often used as a marker for nociceptors but it only identifies a sub-set of these cells. We therefore assayed for the presence of the TTX-resistant sodium channel, $Na_V1.8$, that is restricted to nociceptors (Akopian et al., 1996; Dib-Hajj et al., 1998). Using $Na_V1.8$-specific primers we found that 80 % of capsaicin-responsive neurons analyzed express $Na_V1.8$ mRNA (19 of 24; Fig. 14c, 14e). Most significantly, all neurons that expressed e37a also expressed $Na_V1.8$ (14 of 14; Fig. 14c, 14e) establishing a strong link between a second molecular marker of nociceptors and the presence of $Ca_V2.2$ mRNA containing e37a. Importantly, we next assessed the functional consequences of cell-specific expression of $Ca_V2.2e[37a]$ on the N-type current.

Calcium currents in capsaicin-responsive and non-responsive neurons

We used the perforated, whole-cell patch recording method to record calcium currents from neurons of the dorsal root ganglia (Horn and Marty, 1988). 2 mM calcium was the charge carrier. Capsaicin-responsive neurons were significantly smaller in size on average (18 ± 3 pF), compared to non-responsive neurons (27 pF ± 3) ($p<0.001$) as reported by others

(Cardenas et al., 1995). Initially, the most obvious difference among cells when we separated them on the basis of capsaicin-sensitivity was the presence of a significantly larger low threshold Ca current in non-responsive neurons. This low threshold component is prominent in exemplar current traces and in the average current voltage-relationships from non-responsive neurons (Fig. 15a). In this regard, our findings are consistent with the reports of others (Blair and Bean, 2002; Carbone and Lux, 1984; Cardenas et al., 1995; Schroeder et al., 1990; Scroggs and Fox, 1992a). The high threshold component of the whole cell calcium channel current originates from the activity of additional classes of calcium channel including N-type (Regan et al., 1991; Scroggs and Fox, 1992a). On first inspection, the multi-component, high voltage-activated current appeared similar between capsaicin-responsive and non-responsive neurons (Fig. 15a, activation mid-points for the high voltage-activated currents were close to –15 mV). For this study, we were specifically interested in the N-type current and isolated it from other voltage-activated calcium currents by toxin-subtraction using ω-conotoxin GVIA (ω-Ctx GVIA) (Regan et al., 1991; Scroggs and Fox, 1992b).

N-type currents in capsaicin-responsive and non-responsive neurons

Consistent with other reports, the ω-Ctx GVIA-sensitive, N-type current was the major component of the total Ca current in capsaicin-responsive and non-responsive neurons (~70%; Fig. 15b) (Regan et al., 1991; Scroggs and Fox, 1992a). The N-type current was well isolated in capsaicin-responsive neurons although in non-responsive neurons a small low-threshold current was still present in toxin-subtracted currents (Fig. 15b). The low threshold current runs down relatively rapidly even in the absence of toxin, and this is the most likely explanation for its presence in toxin-subtracted current-voltage relationships of non-responsive neurons (see Methods section for further details). Most importantly, after isolating the N-type current we revealed significant differences in peak N-type channel current densities between capsaicin-responsive 111 ± 12 pA/pF (n = 20) and non-responsive neurons 72 ± 8 pA/pF (n = 9) (Fig. 15b; $p < 0.05$). Although significantly larger, the biophysical properties of N-type currents in capsaicin-responsive neurons were not distinguishable from non-responsive neurons ($V_{½}$ and k values for non-responsive neurons were: -16 ± 2 mV and 5.4 ± 0.6, n = 9, compared to -15 ± 1 mV and 5.2 ± 0.3 for responsive neurons, n = 20; Fig. 15b). Our data suggests that capsaicin-responsiveness is linked to a significantly greater number of functional N-type channels.

Exon 37a is linked to larger N-type current density in capsaicin-responsive neurons

Having demonstrated preferential expression of a unique splice isoform of $Ca_V2.2$ in a sub-set of capsaicin-responsive neurons (Fig. 14b-d), we tested the hypothesis that the presence of $Ca_V2.2e[37a]$ was linked to larger N-type current densities. We selected only capsaicin-responsive neurons, analyzed the N-type current, harvested these same cells, and performed RT-PCR to assay for the presence of each of $Ca_V2.2e[37a]$, $Ca_V2.2e[37b]$, and GAPDH. We performed this complete series of manipulations on 16 individual capsaicin-responsive neurons. These were subsequently separated into two pools, those that expressed $Ca_V2.2e[37a]$ mRNA and those that did not. Fig. 16 summarizes our results and reveals a significant difference in N-type current densities between cells that contain and lack $Ca_V2.2e[37a]$. Capsaicin-responsive neurons that contained and lacked exon 37a were not distinguishable based on cell size, consistent with them representing a relatively homogenous population of nociceptors (20 ± 3 pF, $n = 8$, and 18 ± 1 pF, $n = 8$, for e37a-containing and lacking, respectively). The presence of the $Ca_V2.2e[37a]$ splice isoform was, however, associated with 1.6-fold larger N-type currents (peak current densities were 122 ± 11 pA/pF, $n = 8$, in cells containing e37a compared to 76 ± 3 pA/pF, $n = 8$, in cells lacking e37a; $p < 0.05$; Fig. 16). We also compared the basic biophysical properties of the N-type currents. The voltage-dependence of N-type channel activation, as well as the kinetics of activation and inactivation is not significantly different in cells containing and lacking exon 37a, despite their very different current densities (Fig. 16a-c).

Exon 37a induces larger N-type currents in a non-neuronal expression system

To establish a direct link between expression of the $Ca_V2.2e[37a]$ splice isoform and larger N-type channel current amplitudes, and to eliminate the potential contribution of other factors, we compared $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$ clones expressed in *Xenopus* oocytes under identical conditions. The resultant N-type channel currents were compared in detail and our analysis summarized in Fig. 17. N-type channel currents in oocytes injected with $Ca_V2.2e[37a]$ cRNA were consistently and significantly larger than those in oocytes injected with the same amount of $Ca_V2.2e[37b]$ cRNA (1.6-fold, $p < 0.05$; Fig. 17). Currents were recorded under identical conditions and the same complement of auxiliary subunits co-expressed. These differences between isoforms were observed over a three-day period (see

legend to Fig. 17). We also compared the biophysical properties of the two splice isoforms. The voltage-dependence and kinetics of N-type channel activation were different between $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$ channels. $Ca_V2.2e[37a]$ channels activated at voltages 8 mV more hyperpolarized and with slightly faster kinetics at the same voltages, compared to $Ca_V2.2e[37b]$ (Fig. 17b). To estimate the effect of left shifting the $Ca_V2.2$ channel activation on current amplitude, we plotted the predicted current-voltage relationship for $Ca_V2.2e[37b]$ using the activation curve for $Ca_V2.2e[37a]$ (Fig. 17a, dashed line). An 8 mV shift in activation results in a small increase in peak current because of the greater driving force on ions at hyperpolarized voltages, but this is insufficient to account for the 1.6-fold difference in peak current amplitudes between $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$. We also demonstrated that the voltage-dependence and kinetics of channel inactivation were indistinguishable between splice isoforms (Fig. 17c and see legend). These latter results exclude the possibility that differences in sensitivity to steady-state inactivation between splice isoforms might underlie their different current amplitudes.

Discussion

N-type calcium channels are critical for the transduction of pain in nociceptive neurons of dorsal root ganglia. Here we identify a novel splice form of the N-type $Ca_V2.2$ subunit restricted to dorsal root ganglia, and within this tissue, preferentially expressed in a subset of nociceptive neurons. mRNA levels of this $Ca_V2.2$ splice isoform are substantial and e37a-containing $Ca_V2.2$ mRNA strongly correlates with significantly larger N-type calcium current densities. Cell-specific alternative splicing at the e37a/e37b locus of $Ca_V2.2$ provides a mechanism for increasing N-type current density down-stream of gene transcription.

Functional implications

The crucial role for the N-type channel in nociception is supported by several lines of evidence including the localization of N-type channels at primary synapses of nociceptive neurons in the dorsal horn laminae I/II (Kerr et al., 1988), dominance in controlling neurotransmitter release from presynaptic terminals of nociceptive neurons (Holz et al., 1988; Maggi et al., 1990), impaired nociception in mice lacking $Ca_V2.2$ (Hatakeyama et al., 2001; Kim et al., 2001; Saegusa et al., 2001), and powerful analgesic properties of spinally-administrated N-type channel blockers in a variety of animal pain models (Bowersox et al.,

1996; Bowersox and Luther, 1998; Bowersox et al., 1995; Matthews and Dickenson, 2001; Vanegas and Schaible, 2000). The N-type channel is a target for modulation by many neuromodulators and second messenger signaling pathways. Most neuromodulators, including morphine, couple to the N-type channel to inhibit its activity and down-regulate transmitter release (Ikeda and Dunlap, 1999). Here, we describe a mechanism for increasing the activity of the N-type current in a sub-set of nociceptive neurons that has the potential to increase the coupling efficiency between depolarization and transmitter release, thereby modifying the transmission of noxious stimuli. A characteristic feature of nociceptive neurons is their unusually wide action potentials that have a signature shoulder phase (Blair and Bean, 2002; Djouhri et al., 1998; Dunlap and Fischbach, 1978; Harper and Lawson, 1985; Heyman and Rang, 1985; Ritter and Mendell, 1992). High voltage-activated calcium channels, predominantly N-type, together with TTX-resistant sodium channels are the main contributors to the plateau phase (Baccei and Kocsis, 2000; Blair and Bean, 2002; Renganathan et al., 2001; Scroggs and Fox, 1992b). Cell-specific alternative splicing of e37a in nociceptive neurons also represents a potential mechanism for shaping action potential duration by increasing N-type current density. Although other ion channels clearly remain important, it would be interesting to determine if a correlation exists between levels of e37a and action potential half-widths in single cells. Inclusion of e37a during the processing of $Ca_V2.2$ RNA is cell-specific, raising the possibility that this splicing event might be regulated by specific stimuli or level of neuronal activity. For example, alternative splicing of the STREX exon in the calcium-activated potassium channel, *slo*, is regulated by stress hormones and membrane depolarization (Xie and Black, 2001; Xie and McCobb, 1998). mRNA and protein levels of the calcium channel $Ca_V\alpha2\delta$ subunit protein have been reported to be elevated in dorsal root ganglia in a rat injury model (Luo et al., 2001). The $Ca_V\alpha2\delta$ subunit modifies calcium channel properties and increases channel surface expression (Felix, 1999; Witcher et al., 1993). Although total levels of $Ca_V2.2$ mRNA were reportedly unchanged in the same study (Luo et al., 2001) the splicing pattern at the e37a/e37b locus was not analyzed. Our data suggests that a shift in the splicing pattern from e37bcontaining to e37a-containing $Ca_V2.2$ mRNA could provide a powerful and relatively rapid mechanism, independent of gene transcription, for increasing N-type current density in the pain pathway.

Mechanism of action of exon 37a

Our experiments in *Xenopus* oocytes with cloned $Ca_V2.2$ channels demonstrate a direct link between the presence of exon 37a and significantly larger N-type currents. In this respect our studies of cloned channels parallel nicely those of native N-type currents in capsaicin-responsive neurons. Although other factors need to be ruled out, such as differences in single channel conductance, our results are most consistent with the hypothesis that $Ca_V2.2e[37a]$ splice isoforms produce a greater number of functional N-type channels compared to $Ca_V2.2e[37b]$. Our analysis of $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$ currents recorded from oocytes did, however, also reveal differences in the voltage-dependence and kinetics of N-type channel activation. By contrast, the biophysical properties of native N-type currents isolated by conotoxin-subtraction were not significantly different in capsaicin-responsive cells separated based on the presence and absence of $Ca_V2.2e[37a]$ (Fig. 16a-c). It is possible that the biophysical properties that differ between splice isoforms in the oocyte expression system are present in native currents, but masked in our analysis by a combination of the toxin-subtraction method and the presence of other splice isoforms with different properties (Fig. 10) (Lipscombe et al., 2002). While insufficient alone to account for the different current amplitudes, if present in neurons, such biophysical differences between splice isoforms would combine with the increased effectiveness of $Ca_V2.2e[37a]$ mRNA, to induce larger N-type currents and increase calcium entry through the N-type pathway. Our oocyte experiments demonstrate that the mechanisms underling the different N-type current amplitudes induced by splice isoforms are not unique to neurons, and further, that the information necessary to mediate these differences are contained in the 14 amino acids that are different between $Ca_V2.2e[37a]$ and $Ca_V2.2e[37b]$ (Fig. 13a). The 37a/37b splice site is located in the C-terminus of $Ca_V2.2$, a region implicated in the trafficking and targeting of calcium channels (Gao et al., 2000; Maximov and Bezprozvanny, 2002; Maximov et al., 1999). It is possible that the $Ca_V2.2e[37a]$ isoform is more efficiently targeted to the plasma membrane, although other mechanisms including differences in protein stability cannot be ruled out. The C-terminus of $Ca_V2.2$ constitutes ~25% of the channel protein and is encoded by exons 36-46 (Fig. 10a). It contains several protein binding sites including those that bind G-proteins, Mint1, CASK, and RIM binding proteins (Hibino et al., 2002; Maximov et al., 1999; Simen et al., 2001). However, these sites fall distal to the e37a/e37b locus. Further, all binding studies using recombinant $Ca_V2.2$ protein to date have been performed with the e37b-containing splice form. A comparison of amino acid sequences suggests that e37a modifies a

putative EF-hand motif present in e37b. However, so far we have not observed calcium-dependent differences between splice forms transiently expressed in a mammalian cell line even with low internal calcium buffering. Exons homologous to e37a and e37b are present in the closely related $Ca_V2.1$ gene of the P/Qtype channel and are also predicted to modify a putative EF-hand motif (Soong et al., 2002). The functional significance of splicing at the e37a/e37b locus in $Ca_V2.1$ is not known.

Potential therapeutic implications

N-type channel antagonists that target sequences specific to e37a of $Ca_V2.2$, could offer significant benefits over current N-type blockers such as SNX-111 that have been used with limited success as analgesics because of undesirable side effects. These include inhibition of sympathetic tone, disrupted motor coordination, and dizziness and blurred vision (Bowersox et al., 1996; Brose et al., 1997; Chaplan et al., 1994; Vanegas and Schaible, 2000). Selective inhibition of the e37a splice form of $Ca_V2.2$ represents a potential strategy for targeting N-type currents in a subset of nociceptive neurons while leaving basal transmission mediated by the N-type channel unaffected. It will now be important to determine the sub-cellular distribution of these splice forms of $Ca_V2.2$ in nociceptive neurons using exon-specific antibodies. Other splice forms of the $Ca_V2.2$ subunit arising from alternative splicing at a different locus in the C-terminus have been reported that are differentially targeted in the cell (Maximov and Bezprozvanny, 2002). Cell-specific alternative splicing of other ion channels exists (Black, 1998; Jones et al., 1999; Navaratnam et al., 1997; Rosenblatt et al., 1997) and offers a powerful means to fine-tune the properties of channel activity down-stream of transcription. We demonstrate cell-specific alternative splicing of the N-type Ca channel in a subset of nociceptive neurons and suggest that this offers a mechanism for increasing the efficiency of coupling between membrane depolarization and calcium entry in the pain pathway.

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Each of the foregoing patents, patent applications and references is hereby incorporated by reference.

While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

What is claimed is: